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(54) CELL MEMBRANE-DIRECTED DRUGS

(57) Drugs wherein peptides having an affinity specific to phospholipids, preferably those which constitute a lipid bilayer forming a cell cortex and the content of which in the lipid bilayer increases in abnormal cells, for example, injured, denatured or activated cells are bonded to physiologically active substances; deoxyribonucleic acids (DNA) encoding the amino acid sequences of the drugs when the physiologically active substances are peptides; and processes for producing these drugs. These drugs and novel peptides are useful as preventives and remedies for diseases accompanied by blood coagulation disorders, inflammation and immune responses.

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Description

Technical Field

[0001] This invention relates to drugs in which peptides having affinity specific for phospholipids, preferably those which are contained in the constituents of lipid bilayers forming the surface layers of cells and of which the proportion in the outer part of each lipid bilayer increases when the cell is not normal, for example, in the case where it is damaged, denatured or activated, and biologically active substances bind to each other, deoxyribonucleic acid (DNA) which codes for the amino acid sequence of the drug in the case where the biologically active substance is a peptide, and processes for producing such drugs.

[0002] The invention also relates to a novel peptide having affinity specific for a phospholipid, preferably one which is contained in the constituents of lipid bilayers forming the surface layers of cells and of which the proportion in the outer part of each lipid bilayer increases when the cell is not normal, for example, in the case where it is damaged, denatured or activated, more preferably phosphatidylserine or phosphatidylethanolamine, further preferably phosphatidylserine, and DNA coding for the peptide.

[0003] The drugs and novel peptides of the invention are useful as preventives and therapeutics of diseases involving coagulopathy, inflammations and immune response.

Background Art

[0004] Active studies are conducted today in connection with the creation of new drugs of high utility and as one of such studies an attempt is known that is directed to enhance the proportion in which a drug administered *in vivo* is delivered to the site for effective action while reducing the amount in which it becomes ineffective. This is an attempt at delivery of the drug in a site-specific manner, or an attempt of the so-called "targeting". A representative method of targeting is one that utilizes an antigen-antibody reaction. A method that may be mentioned as an example of this approach is one in which an F(ab')₂ fragment of a tumor cell specific antibody (21B2) is bound to liposomes containing adriamycin (ADM) to prepare immunoliposomes such that ADM is delivered on tumor cells [I. Uyama et al., Jpn. J. Cancer Res., Vol. 85, 434 (1994)]. Another representative method of targeting is one that utilizes the interaction between a polypeptide-composed receptor and its ligand. A method that may be mentioned as an example of this approach is one in which an RGD polypeptide sequence having an ability to bind to a GPIIb/GPIIIa receptor is attached to the C terminus of phospholipase A₂ (PLA₂), whereby the PLA₂ is delivered on the membrane surface of platelets [A.C.A.P.A. Bekkers et al., Thrombosis and Haemostasis, Vol. 74, 1138 (1995)]. Other examples that have so far been reported include one in which a peptide having affinity for heparin present on the surface of cells is bound to superoxide dismutase (SOD) or complement regulatory proteins such that the SOD is delivered on the cell surface [M. Inoue et al., J. Biol. Chem., Vol. 266, 16409 (1991)] or the complement regulatory proteins is delivered on the cell surface (International Patent Publication WO/96/34965).

[0005] Peptides are known which themselves have no biological activity but which have selective affinity to the target site, whereby the binding of a biologically active substances to the target site of action is inhibited through a competitive reaction. An example of such peptides is a polypeptide that binds to a receptor on the surface of cells selectively, thereby inhibiting a ligand from binding to the receptor. An example of such polypeptides is a polypeptide fragment derived from the antigen recognizing site (or complementary determining region: CDR) of an anti-TNF α monoclonal antibody, which inhibits TNF α from binding to a receptor [E. Doring et al., Molecular Immunology, Vol. 31, 1059 (1994)]. Another example is a polypeptide which, by selective binding to a phospholipid, phosphatidylserine, inhibits a factor involved in the progress of a blood coagulation from binding to the phospholipid and which is composed of 30 amino acids at the terminus of the C2 region of a human factor VIII (International Patent Publication WO/90/15615) or 12 amino acids derived from the CDR of a phosphatidylserine recognizing antibody (Japanese Patent Public Disclosure KOKAI No. 92992/1993).

[0006] Another example that concerns the creation of new drugs of high utility is an attempt at imparting a new biologically active function to an already biologically active peptide by genetic engineering techniques. As regards TM which is known to suppress a blood clotting reaction, reports have been made of a TM derivative which has a fibrinolytic enzyme such as a tissue plasminogen activator (tPA) bound to a TM peptide (Japanese Patent Domestic Announcement KOHYO 505554/1992) and a TM derivative which has a specified amino acid sequence bound to the C terminus of a TM peptide so as to impart its action in enhancing the activity of antithrombin III and suppressing platelet aggregation (Japanese Patent Public Disclosure KOKAI No. 279497/1994).

Disclosure of Invention

[0007] There are known many drugs today that must be used in limited doses because of the side effects they have.

For example, heparin and antithrombin III are used as anticoagulants but the use of these substances must occasionally be limited since they have side effects such as the tendency to cause bleeding.

[0008] When administering drugs, it is generally required to administer more than a certain dose in order to attain a specified efficacy and side effects to the patient are sometimes a serious problem. In addition, it is by no means rare that administering high doses of drugs will eventually often increase the economic burden on the part of the patient. Therefore, it is desired to develop drugs that have high enough activity to exhibit satisfactory efficacy in low doses such that the side effects from drug administration can be reduced and which can be used more extensively without increasing the economic burden on the patient.

[0009] Under the circumstances, the present inventors conducted intensive studies with a view to attaining the stated object. As a result, they found that a drug having affinity for a specified phospholipid that was created by binding a biologically active substance to a substance having affinity for the specified phospholipid had an improved ability to localize on the specified phospholipid, thereby achieving a marked enhancement of its action and efficacy. The inventors further found that this marked enhancement of the action and efficacy of the drug was dependent on the specified phospholipid, which led to the accomplishment of the present invention.

[0010] Thus, a first aspect of the present invention is a drug that contains both a substance having affinity for a phospholipid and a biologically active substance. The drug is preferably one having a novel substance that possesses both a portion having affinity for a desired phospholipid and a portion having biological activity; since the drug is obtained as a different form than it inherently occurs in nature, it is a drug having a chimeric substance or a substance produced by fusion of different proteins. Herein, said affinity for a phospholipid or said portion having affinity for a phospholipid preferably originates from the substance having affinity for a phospholipid, whereas the biological activity or the portion having biological activity preferably originates from the biologically active substance. Specifically, the drug is one that contains both a substance having affinity for a specified phospholipid, preferably phosphatidylserine or phosphatidylethanolamine, more preferably phosphatidylserine, and a biologically active substance. Herein, the substance having affinity for a specified phospholipid is preferably a peptide or a peptide-containing substance. Said peptide has a sequence represented by the following general formula; preferably, it is composed of said sequence. It should be noted that all of the sequences to be described in this specification permit substitution, deletion, addition, insertion and so forth in part of their constituent elements unless their characteristics are impaired. Specifically, if the sequence is an amino acid sequence, one or more amino acids may be substituted, deleted, added, inserted or otherwise modified without impairing the activity of the sequence.

$(A1)n_1 - (A2)n_2 - (A3)n_3$

(provided that in the general formula set forth above, A1 represents the amino acid sequence denoted by Sequence ID No. 1 or 10; A2 and A3 represent the amino acid sequences denoted by Sequence ID Nos. 2 and 3, respectively; n_1 , n_2 and n_3 which represent the repeating numbers of A1, A2 and A3, respectively, are 0 - 5, 1 - 5 and 0 - 5, respectively; preferably, n_1 is 0 or 1, n_2 is 1, 2 or 3, and n_3 is 0 or 1).

[0011] More preferably, the substance having affinity for a phospholipid has at least either of the following sequences:

$(A1)_1 - (A2)_1 - (A3)_1$ (Sequence ID No. 8 or 9)

$(A2)_1 - (A3)_1$ (Sequence ID No. 5)

$(A2)_2 - (A3)_1$ (Sequence ID No. 6)

$(A2)_3 - (A3)_1$ (Sequence ID No. 7)

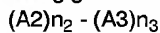
$(A2)_2$ (Sequence ID No. 22)

[0012] The drug according to the first aspect of the present invention preferably contains a peptide as the biologically active substance; specifically, at least one member selected from the group consisting of a factor involved in a blood coagulation system, a factor involved in a fibrinolytic system, a factor involved in an immune response reaction, a factor

suppressing cytopathy, a factor inhibiting the activity of proteases and modified versions thereof, more preferably, at least one member selected from the group consisting of a factor suppressing a blood coagulation, a factor enhancing a fibrinolytic system, a factor suppressing a complement activating reaction, a factor suppressing the cytopathy due to active oxygen, a factor inhibiting the activity of proteases and modified versions thereof, further preferably at least one member selected from the group consisting of TM, the second region of UTI, MCP, UTI and modified versions thereof, particularly preferably a peptide having the amino acid sequence denoted by Sequence ID No. 4 or either of Sequence ID Nos. 23 - 25.

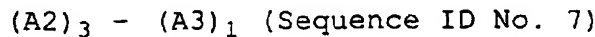
[0013] In a mode of containing both the biologically active substance and the substance having affinity for a specified phospholipid, the drug according to the first aspect of the invention preferably contains a linkage via a peptide bond, more preferably a linkage in which the N terminal amino acid of the substance having affinity for a phospholipid and the C terminal amino acid of the biologically active substance or, alternatively, the C terminal amino acid of the substance having affinity for a phospholipid and the N terminal amino acid of the biologically active substance are associated by a peptide bond, further preferably, a mode in which the N terminal amino acid of the substance having affinity for a phospholipid and the C terminal amino acid of the biologically active substance are associated by a peptide bond.

[0014] In its second aspect, the present invention provides a peptide having the amino acid sequence represented by the following general formula:



provided that A2 and A3 are the amino acid sequences denoted by Sequence ID Nos. 2 and 3, respectively; n_2 which is the repeating number of A2 is 2 or 3; n_3 which is the repeating sequence of A3 is 0 or 1.

[0015] More preferably, the amino acid sequence of said peptide has either of the following sequences:



[0016] According to its third aspect, the present invention provides DNA coding for the amino acid sequence of the drug according to the first aspect of the present invention or the peptide portion of said drug.

[0017] According to its fourth aspect, the present invention provides DNA coding for the amino acid sequence of the peptide according to the second aspect of the invention.

[0018] According to its fifth aspect, the present invention provides a process for producing the drug according to the first aspect of the invention.

Brief Description of the Drawings

[0019]

Fig. 1 is a diagram showing the DNA primers used in PCR for yielding the DNA of the present invention.

Fig. 2 is a diagram showing the DNA primers used in PCR for yielding the DNA of the present invention.

Fig. 3 is a diagram showing the process of cloning human TM cDNA and constructing a human TM expressing vector.

Fig. 4 is a diagram showing synthetic DNAs used in constructing a vector expressing soluble human TM having affinity for phosphatidylserine, a vector expressing the second region of human UTI having affinity for phosphatidylserine, a vector expressing human UTI having affinity for phosphatidylserine and a vector expressing soluble human MCP having affinity for phosphatidylserine according to the present invention.

Fig. 5 is a diagram showing other synthetic DNAs also used in constructing a vector expressing soluble TM having affinity for phosphatidylserine, a vector expressing the second region of human UTI having affinity for phosphatidylserine, a vector expressing human UTI having affinity for phosphatidylserine and a vector expressing soluble human MCP having affinity for phosphatidylserine according to the present invention.

Fig. 6 is a diagram showing the process of constructing an expression vector pM1350 according to the present invention.

Fig. 7 is a diagram showing the process of constructing an expression vector pM1357 according to the present invention.

Fig. 8 is a diagram showing the process of constructing an expression vector pM1356 according to the present invention.

Fig. 9 is a diagram showing the process of constructing a vector pM1354 according to the present invention.

Fig. 10 is a diagram showing the process of constructing an expression vector pM1355 according to the present invention.

Fig. 11 is a graph showing the result of comparison of various kinds of soluble human TM having affinity for phosphatidylserine according to the present invention with unmodified soluble human TM in terms of the ability to accelerate protein C activation, in which the vertical axis plots the relative activity of action in accelerating the protein C activation.

Fig. 12 is a graph showing that a soluble human TM (rsTMC2) having affinity for phosphatidylserine according to the present invention is capable of accelerating protein C activation in a phosphatidylserine specific manner, with the open columns referring to unmodified soluble human TM (rsTM) and the black columns to the soluble human TM (rsTMC2) having affinity for phosphatidylserine, in which PC, PA, PE and PS on the horizontal axis signify the components of liposomes and represent phosphatidylcholine, phosphatidic acid, phosphatidylethanolamine and phosphatidylserine, respectively, and Lipo(-) signifies the absence of liposomes, whereas the vertical axis plots the relative activity of the action in accelerating protein C activation.

Fig. 13 is a graph showing that soluble human TMs having affinity for phosphatidylserine according to the present invention bind to phospholipid in a phosphatidylserine specific manner, in which the open columns refer to unmodified soluble human TM (rsTM) whereas the hatched and black columns refer to the soluble human TMs having affinity for phosphatidylserine (which are respectively rsTMTd and rsTMC2), and the horizontal axis of the graph plots the content of phosphatidylserine in liposomes composed of phosphatidylcholine and phosphatidylserine whereas the vertical axis plots the amount of binding (absorbance).

Fig. 14 is a diagram showing the process of constructing an expression vector pM1358 according to the present invention.

Fig. 15 is a diagram showing the process of constructing an expression vector pM1213 according to the present invention.

Fig. 16 is a diagram showing the process of constructing an expression vector pM1380 according to the present invention.

Fig. 17 is a diagram showing the process of constructing an expression vector pM1370 according to the present invention.

Fig. 18 is a diagram showing the process of constructing an expression vector pM1371 according to the present invention.

Fig. 19 is a diagram showing the result of comparison of human UTI having affinity for phosphatidylserine according to the present invention with unmodified human UTI in terms of the ability to suppress active oxygen production, in which the vertical axis plots the amount of production of active oxygen (the rate of change in absorbance per unit time).

Fig. 20 is a graph showing the result of comparison of the second region of human UTI having affinity for phosphatidylserine according to the present invention (rR-020C2, indicated by ● in the graph) with the second region of human UTI expressed in *E. coli* (indicated by ○ in the graph) in terms of the ability to inhibit the activity of prothrombinase, in which the horizontal axis plots the concentrations of rR-020C2 and the second region of *E. coli* expressed human UTI in terms of trypsin inhibiting activity whereas the vertical axis plots the percent residual activity of prothrombinase.

Fig. 21 is a graph showing that the second region of human UTI (rR-020C2) having affinity for phosphatidylserine according to the present invention has the ability to inhibit the activity of prothrombinase in a phosphatidylserine specific manner, with the open columns referring to rR-020 and the hatched columns R-020C2, and the horizontal axis of the graph plotting the content of phosphatidylserine in liposomes composed of phosphatidylcholine and phosphatidylserine whereas the vertical axis plots the percent inhibition of the activity of prothrombinase.

Fig. 22 is a diagram showing the process of constructing an expression vector pM1390 according to the present invention.

Best Mode for Carrying Out the Invention

[0020] On the pages that follow, the present invention will be described in detail.

[0021] The "phospholipid" as used in the present invention is a desired phospholipid that is intended for targeting a biological substance to a specified site and which is contained in the constituent components of the lipid bilayers forming the surface layers of cells such that the proportion of its content in the outer part of each lipid bilayer will increase when the cells are not normal, for example, in the case where they are damaged, denatured or activated. More specifically, the phospholipid is such that its content increases in such cases as where a blood coagulation is in progress, the

so-called immune response reactions of cells such as their activation, impairment and/or apoptosis due to inflammation or immunocytes are in progress, a cell impairing reaction due to active oxygen is in progress or where a cell activating and/or impairing reaction due to proteases is in progress. As typical examples of such phospholipid, phosphatidylserine and phosphatidylethanolamine [Alan J. Schroit et al., *Biochim. Biophys. Acta*, Vol. 1071, 313 (1991)] may be mentioned, and phosphatidylserine is preferred.

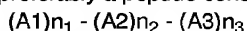
[0022] The term "having affinity" as used in the present invention refers to the performance of a certain interaction. The term "interaction" includes mutual binding, formation of a complex, mutual recognition of molecules, tendency to move and/or aggregate in a specified direction, causing the shape of molecules to change, and mutual reaction. In the case of mutual binding, the mode of binding is in no way limited and it may be non-covalent bonding typified by electrostatic bonding and hydrophobic bonding or covalent bonds typified by a disulfide bond, an ester bond, an ether bond and a peptide bond.

[0023] Thus, the first aspect of the present invention is a substance or a composition, preferably a drug, that contain both a substance having affinity for a phospholipid and a biologically active substance, and which are characterized in that their action and efficacy are enhanced under such a condition that a specified phospholipid is present.

[0024] The term "under such a condition that a phospholipid is present" as used herein includes not only artificially created environments but also natural environments. Artificially created environments include the state where phospholipids, blood, cells, living tissues and their disrupted products are contained *in vitro* or within containers such as test tube whereas natural environments include all *in vivo* parts such as blood vessels, brain and other organs.

[0025] It should also be noted that the "peptide" as used in the present invention is in no way limited in the length of its amino acid sequence and covers the range from so-called dipeptides consisting of two amino acids to polypeptides consisting of 1,000 or more amino acids.

[0026] The substance having affinity for a phospholipid as used in the present invention is in no way limited in the molecules it is composed of or its shape as long as it has affinity for a specified phospholipid. Preferably, it is a peptide, more preferably a peptide consisting of the following amino acid sequence:



where A1 is the amino acid sequence denoted by Sequence ID No. 1 or 10, and A2 and A3 are the amino acid sequences denoted by Sequence ID Nos. 2 and 3, respectively. It should be noted that A1 may contain all or part of the amino acid sequence of Sequence ID No. 1. For example, A1 may contain the amino acid sequence denoted by Sequence ID No. 10 but it preferably contains all of the amino acid sequence of Sequence ID No. 1. Symbol Xaa in Sequence ID No. 2 is Thr or Leu. It should also be noted that A3 may contain part or all of the amino acid sequence of Sequence ID No. 3. Referring to n_1 , n_2 and n_3 , they are any numbers representing the repeating numbers of the amino acid sequences A1, A2 and A3, respectively; n_1 is preferably 5 or less, more preferably 0 or 1, n_2 is preferably at least 1, more preferably 1, 2 or 3, and n_3 is preferably 5 or less, more preferably, 0 or 1.

[0027] The method of combining the sequences set forth above is not limited in any way as long as the stated peptide has affinity for a specified phospholipid. Advantageous examples to be combined include the sequences denoted by Sequence ID Nos. 5 - 9 and 22.

[0028] It should, however, be noted that the aforementioned amino acid sequences are illustrative only and the peptide having affinity for a specified phospholipid according to the present invention is in no way limited in its amino acid sequence as long as it has affinity for a specified phospholipid and depending on the need, the aforementioned amino acid sequences may be subjected to substitution, deletion, insertion, addition and so forth. Further, they may be modified as required. Alternatively, peptides having affinity for phosphatidylserine that consist of entirely different amino acid sequences from the aforementioned ones may be substituted, for example, a peptide derived from a Gla region (characterized by γ -carboxyglutamic acid residue) which has affinity for the phosphatidylserine contained in a coagulation-related factor such as factor Xa [Mann K.G. et al., *Blood*, Vol. 76, 1 (1990)], a peptide having affinity for the phosphatidylserine derived from factor V [Thomas L.O. et al., *J. Biol. Chem.*, Vol. 267, 4189 (1992)], a peptide having affinity for the phosphatidylserine derived from Annexin V [M.A. Swairjo et al., *Nature Struct. Biol.*, Vol. 2, 968 (1995)], and so forth or derivatives thereof.

[0029] The "biologically active substance" in the drug of the present invention may be of any substance that exhibits a pharmacological action *in vivo* and it may include a peptide having biological activity, a chemical substance exhibiting a pharmacological action, as well as their aggregates, encapsulations and so forth, a peptide with being preferred. The "peptide having biological activity" mentioned herein may be any peptide that is involved in *in vivo* reactions and it may be modified, as required. Preferably, the biologically active substance is a peptide which inherently makes a certain mutual interaction with cell membranes by itself or a peptide that interacts with substances that occur within cells, on cell membranes, in the surface layers of cells or their surroundings; modified versions, variants and derivatives of such peptides are also included. If the biologically active substance is an inherently non-water-soluble peptide, as exemplified by some membrane proteins, it may optionally be modified to a water-soluble form. Advantageous examples of such peptides include a factor involved in a blood coagulation system, a factor involved in a fibrinolytic system, a factor involved in immune response, a factor suppressing cytopathy, a factor inhibiting the activity of proteases and so forth,

more preferably, a factor suppressing a blood coagulation system, a factor enhancing a fibrinolytic system, a factor suppressing a complement activating reaction, a factor suppressing the cytopathy due to active hydrogen, a factor inhibiting the activity of proteases and so forth. To illustrate in greater detail, examples of the factor involved in a blood coagulation system and/or the factor suppressing a blood coagulation system include TM, the second region of UTI, antithrombin III (ATIII), a tissue factor pathway inhibitor (TFPI) and so forth, as well as modified versions, variants and derivatives of these factors. Examples of the factor involved in a fibrinolytic system and/or the factor enhancing a fibrinolytic system include tPA, urokinase (UK) and so forth, as well as modified versions, variants and derivatives of these factors. Examples of the factor involved in immune response reactions include complement regulatory proteins such as MCP and a decay-accelerating factor (DAF) which are factors that suppress a complement activating reaction, UTI which inhibits immunocyte-derived proteases and the second region of UTI, as well as modified versions, variants and derivatives of these factors. Examples of the factor suppressing the cytopathy due to active oxygen and/or the factor inhibiting the activity of proteases include UTI, the second region of UTI, elafin, a secretory leukoprotease inhibitor (SLPI) and so forth, as well as modified versions, variants and derivatives of these factors. Other examples of the factor suppressing the cytopathy due to active oxygen include SOD, catalase and so forth, as well as their modified versions, variants and derivatives (i.e., factors having an active oxygen scavenging action). Among these advantageous examples, TM, the second region of UTI, MCP and UTI, as well as their modified versions, variants and derivatives are preferred, and those peptides which are represented by Sequence ID Nos. 4 and 23 - 25 are particularly preferred. These are illustrative only and will in no way limit the biologically active substance to be used in the present invention.

[0030] In the drug according to the first aspect of the present invention, the "chemical substance exhibiting a pharmacological action" is a substance selected from among all substances that exhibit pharmacological actions but which exclude peptides having biological activity and as long as it exhibits a pharmacological action in vivo, it will in no way be limited by the molecular formula with which it is denoted. Specifically, examples include artificially synthesized compounds, chemical substances obtained by separation from natural products and microorganism-produced substances, nucleic acids, saccharides, lipids and so forth, as well as their modified versions. To illustrate in greater detail, cyclophosphamide which is an immunosuppressive substance, actinomycin D which is an anti-cancer agent, an antisense oligonucleotide, hyaluronic acid and lecithin may be mentioned; it should be noted that these are merely intended for illustrative purposes and will in no way limit the biologically active substance to be used in the present invention.

[0031] The term "aggregate" of the substance having biological activity or the compound exhibiting a pharmacological action covers the substance having biological activity or the compound exhibiting a pharmacological action which have been assembled in a quantity greater than a specified level due, for example, to chemical bonding or physical adhesion, and the term "encapsulation" of the substance having biological activity or the compound exhibiting a pharmacological action covers the substance having biological activity or the chemical compound exhibiting a pharmacological action which are incorporated within liposomes, microcapsules or high-molecular weight matrices and so forth.

[0032] The mode in which both the substance having affinity for a phospholipid and the biologically active substance are contained according to the first aspect of the present invention is not limited in any particular way. A preferred mode is such that the substance having affinity for a phospholipid is substantially integral with the biologically active substance to form the drug of the present invention without causing a complete compromise in the affinity for a specified phospholipid which is possessed by the substance having affinity for a phospholipid or in the activity of the biologically active substance. As long as the substance having affinity for a phospholipid is substantially integral with the biologically active substance to form the drug of the present invention without causing a complete compromise in the affinity for a specified phospholipid which is possessed by the substance having affinity for a phospholipid or in the activity of the biologically active substance, all possible modes such as a mixture, a composition, a complex, a bound form and so forth are included. Thus, the two substances may be simply mixed with each other or they may be contained in a composition or, alternatively, they may perform interaction with each other. The term "interaction" covers mutual binding, formation of a complex, mutual recognition of molecules, tendency to move and/or aggregate in a specified direction, causing the state of molecules to change and mutual reaction, with direct or indirect bonding in either of these forms being preferred. In the case of mutual binding, the mode of binding is in no way limited and it may be non-covalent bonding typified by electrostatic bonding and hydrophobic bonding or covalent bonding typified by a disulfide bond, an ester bond, an ether bond and a peptide bond. Depending on the need, a suitable linker and an adapter such as a peptide having any amino acid sequence or any compound may be interposed, as exemplified by a biotin-avidin bond, an antibody-antigen bond or a bond formed by a receptor and its ligand; further, modifications may be applied as required. If at least one of the biologically active substance and the substance having affinity for a phospholipid is a peptide, examples of the binding site in the peptide include an amino group, a carboxyl group and a thiol group in a cysteine residue that are present at the N terminus, C terminus or inside chains, with the N- or C terminus being preferred. In a particular case where both the biologically active substance and the substance having affinity for a phospholipid are peptides, it is preferred to contain a bond established by a peptide bond and a more preferred mode is such that the N terminus of either one of the biologically active substance and the substance having affinity for a phospholipid is bound to the C terminus of the other peptide via a peptide bond. Thus, chimeric proteins and fused proteins may be mentioned as typical

examples. Among these cases, the one in which the N terminus of the substance having affinity for a phospholipid is linked to the C terminus of the biologically active substance via a peptide bond is particularly preferred. To give a specific preferred example, a drug according to the present invention may be mentioned that contains a peptide composed of an amino acid sequence in which an amino acid sequence selected from among Sequence ID Nos. 5 - 9 and 22 is linked to the C terminus of an amino acid sequence selected from among Sequence ID Nos. 4 and 23 - 25.

[0033] The present invention also provides a novel peptide having affinity for phosphatidylserine which consists of or contains the amino acid sequences denoted by Sequence ID Nos. 6, 7 and 22, as well as a pharmaceutical that contains said peptide as a component. Said pharmaceutical includes one in which the novel peptide having affinity for phosphatidylserine according to the present invention is mixed with or bound to a biologically active substance or its aggregate or encapsulation. The biologically active substance with or to which the novel peptide having affinity for phosphatidylserine has mixed or bound, or the aggregate or encapsulation of the substance has such a nature that it will be delivered selectively on the surface of cells that are not normal, as exemplified by damaged, denatured or activated cells. Stated more specifically, a biologically active substance to which the novel peptide having affinity for phosphatidylserine according to the present invention has bound, optionally via a suitable linker, has such a nature that it will be delivered selectively on the surface of cells that are not normal, as exemplified by damaged, denatured or activated cells. The novel peptide having affinity for phosphatidylserine according to the present invention may be modified with a substance having affinity for the constituent components of the skeleton of an encapsulation of a biologically active substance and subsequently mixed or reacted with the encapsulation of the biologically active substance to produce an encapsulation containing the biologically active substance to the surface of which the novel peptide having affinity for phosphatidylserine has bound and this encapsulation similarly has such a nature that it will be delivered selectively on the surface of cells that are not normal, as exemplified by damaged, denatured or activated cells. To illustrate, if the biologically active substance is contained in liposomes, the novel peptide having affinity for phosphatidylserine according to the present invention is modified with a suitable phospholipid such as phosphatidylethanolamine and thereafter mixed with liposomes to produce a drug in which the novel peptide having affinity for phosphatidylserine according to the present invention has bound to the surface layers of the liposomes and the drug has such a nature that it will be delivered selectively on the surface of cells that are not normal, as exemplified by damaged, denatured or activated cells.

[0034] The novel peptide having affinity for phosphatidylserine according to the present invention can also be used as a drug with which a biologically active factor that is involved in the progress of a blood coagulation is inhibited from binding to the target site of action by a competitive reaction.

[0035] The DNA of the present invention may be of any kind as long as it has, in effect, a DNA sequence coding for the peptides of the substance having affinity for a phospholipid and/or the biologically active substance which compose the drug of the present invention. More specifically, the present invention provides DNA which contains, preferably consists of, the following sequences:

$$(J1) - (D1)m_1 - (D2)m_2 - (D3)m_3 - (J1)$$

where J1 is a DNA sequence coding for the amino acid sequence of a peptide having biological activity and at least either one of 5'- or 3' terminus will be sufficient; D1, D2 and D3 are DNA sequences coding for the peptides represented by A1, A2 and A3, respectively, with D1 being the DNA sequence denoted by Sequence ID No. 11 or 12, D2 the DNA sequence denoted by Sequence ID No. 13 or 14 and D3 the DNA sequence denoted by Sequence ID No. 15. It should be noted that D1 may contain all or part of the DNA sequence of Sequence ID No. 11 to such an extent that there will be no change in its translational frame. For instance, D1 may contain the DNA sequence of Sequence ID No. 12 and it preferably contains all of the DNA sequence of Sequence ID No. 11. Symbol M in Sequence ID Nos. 13 and 14 signifies A or C. It should also be noted that D3 may contain part or all of the DNA sequence of Sequence ID No. 15 to such an extent that there will be no change in its translational frame. Symbols m_1 , m_2 and m_3 are any numbers that represent the repeating numbers of the DNA sequences, D1, D2 and D3, respectively; m_1 is preferably 5 or less, more preferably 0 or 1, m_2 is preferably at least 1, more preferably 1, 2 or 3, and m_3 is preferably 5 or less, more preferably 0 or 1. The method of combining the stated sequences is in no way limited as long as the peptides composed of the amino acid sequences translated from the stated DNA provide the drug of the present invention. To give advantageous examples, J1 may be DNA coding for a factor involved in a blood coagulation, a factor involved in a fibrinolytic system, a factor involved in immune response, a factor suppressing cytopathy, a factor inhibiting the activity of proteases and so forth, more preferably, a factor suppressing a blood clotting reaction, a factor enhancing a fibrinolytic system, a factor suppressing a complement activating reaction, a factor suppressing the cytopathy due to active oxygen, a factor inhibiting the activity of proteases and so forth. To illustrate in greater detail, examples of the factor involved in a coagulation system reaction and/or the factor suppressing a blood coagulation include TM, the second region of UTI, ATIII, TFPI and so forth, as well as modified versions, variants and derivatives of these factors. Examples of the factor involved in a fibrinolytic system and/or the factor enhancing a fibrinolytic system include tPA, UK and so forth, as well as modified versions, variants and derivatives of these factors. Examples of the factor involved in an immune response reactions include complement regulatory proteins such as MCP and DAF which are factors suppressing a complement activating

reaction, UTI which inhibits immunocyte-derived proteases and the second region of UTI, as well as modified versions, variants and derivatives of these factors. Examples of the factor suppressing the cytopathy due to active oxygen and/or the factor inhibiting the activity of proteases include UTI, the second region of UTI, SLPI and so forth, as well as modified versions, variants and derivatives of these factors. Other examples of the factor suppressing the cytopathy due to active oxygen include SOD, catalase and so forth, as well as their modified versions, variants and derivatives (i.e., factors act as active oxygen scavenger). A typical example of a more preferred combination of sequences may be a DNA sequence consisting of nucleotide sequences such that a nucleotide sequence of Sequence ID Nos. 16 - 20 or No. 26 is linked to the 3' end of a nucleotide sequence of Sequence ID No. 21 or Nos. 27 - 29.

[0036] Advantageous examples of the DNA according to the fourth aspect of the present invention which codes for the amino acid of the novel peptide having affinity for phosphatidylserine are the DNA sequences of Sequence ID Nos. 17, 18 and 26 (which code for the peptides of Sequence ID Nos. 6, 7 and 22, respectively).

[0037] The present invention also provides a recombinant DNA, such as a plasmid or expression vector, that contains the DNA of the invention.

[0038] As is well known in the art, in accordance with the degeneracy of genetic codes, at least one base in the DNA gene sequence which encodes polypeptide can be replaced by another base without changing its amino acid sequence. Hence, the DNA of the present invention may have base sequences having one or more base replacements based on the degeneracy of genetic codes. Particularly in the case of producing the peptide of the present invention by genetic engineering techniques, the DNA may have sequences having one or more base replacements in order to provide codons that will be used with high frequency in specified host cells. It should be noted that in the present invention, the sequence of DNA is described starting from the 5' terminal end. In the present invention, A, G, C and T stand for deoxyadenylic acid, deoxyguanylic acid, deoxycytidylic acid and thymidylic acid, respectively.

[0039] The drug of the present invention can be obtained by a process in which the substance having affinity for a specified phospholipid and the biologically active substance are prepared individually and then mixed or bound to each other. Herein, the substance having affinity for a specified phospholipid may be mixed with or bound to the biologically active substance by any method as long as the substance having affinity for a specified phospholipid is substantially integral with the biologically active substance to form the drug of the present invention without causing a complete compromise in the affinity for a specified phospholipid which is possessed by the substance having affinity for a specified phospholipid or in the activity of the biologically active substance.

[0040] The substance having affinity for a specified phospholipid according to the present invention is preferably a peptide and can be produced by a process characterized in that at least one of the following steps is performed:

- a) the step of obtaining said peptide by chemical synthesis;
- b) the step of obtaining DNA having a sequence coding for the amino acid sequence of said peptide;
- c) the step of incorporating said DNA into a vector so as to give a replicable recombinant DNA containing said DNA;
- d) the step of transforming a host cell with said recombinant DNA to give a transformant capable of expressing said peptide; and
- e) the step of culturing said transformant such as to produce said peptide and recovering said peptide from the culture mixture.

[0041] The chemical synthesis method for producing a peptide having a specified amino acid sequence according to the present invention may typically be implemented by using an automatic peptide synthesizer.

[0042] The DNA having a sequence coding for the peptide of the present invention may typically be prepared in the following manner. Unless otherwise expressly stated, general genetic engineering techniques can be implemented on the basis of the procedures described in literature [such as "Molecular Cloning, A LABORATORY MANUAL", Second Edition, T. Maniatis et al., Cold Spring Harbor Laboratory Press (1989)]. To begin with, cDNA prepared on the basis of mRNA extracted from human cells or organs or, alternatively, a commercially available human cDNA library or human chromosomal DNA is used as template DNA. Then, by referring to the known DNA sequence (e.g., the sequence of human factor VIII) and using a DNA probe chemically synthesized with an automatic DNA synthesizer, the template DNA is screened to obtain DNA coding for the desired polypeptide.

[0043] The DNA coding for a polypeptide having affinity for a specified phospholipid can also be obtained solely by chemical synthesis using an automatic DNA synthesizer. Another preferred method for obtaining the DNA coding for a peptide having affinity for a specified phospholipid is one utilizing a polymerase chain reaction (hereinafter designated as PCR). Briefly, by referring to the known DNA sequence (e.g., the sequence of human factor VIII), a DNA primer is chemically synthesized with optional base sequences and restriction enzyme recognizing sites being attached as required and PCR is performed using the above-mentioned cDNA as a template DNA such that the desired DNA is obtained. It should be noted that PCR can be performed by making reference to literature [e.g., "PCR Protocols, A Guide to Methods and Applications", Michael A.I. et al., Academic Press (1990)].

[0044] The step of incorporating the DNA of interest into a vector can be implemented in accordance with the general

genetic engineering techniques described in the stated literature. Briefly, cloning sites in the vector are digested with suitable restriction enzymes and the DNA of interest, optionally after digestion with restriction enzymes, is inserted between the digested cloning sites, optionally with linkers and so forth. The vector to be used herein may be of any kind as long as it is capable of replication within the host to be used but it is preferred to select a vector that contains a promoter, a ribosome binding site, sequence of a signal peptide and/or a poly(A)⁺ signal, all being necessary for expressing the peptide of interest within a host, and which is replicable within the host to be used. As the promoter, ribosome binding site, sequence of signal peptide and poly(A)⁺ signal that are to be used, all promoters, ribosome binding sites, sequence of signal peptide and poly(A)⁺ signals that will function within the host to be used are applicable and these can be synthesized chemically or are available from any cells, host to be used, virus, plasmid or phage and so forth.

[0045] The step of introducing the resulting recombinant DNA into a host cell can be implemented in accordance with the methods customary in the art concerned and which are described in literature (e.g. "Shin Saibo Kogaku Jikken Purotokol", edited by Department of Oncology, Institute of Medical Science, The University of Tokyo, published by Shunjusha, 1991), as exemplified by a competent cell procedure, a calcium phosphate procedure, a DEAE dextran procedure or electroporation and so forth. The host cell into which the resulting recombinant DNA is to be introduced may be eukaryotic as typified by Hela cell, COS cell, CHO cell, yeasts and insect cells or prokaryotic as typified by *Escherichia coli* and *Bacillus subtilis* and any cell that is suitable for expressing the peptide of the present invention may be selected as appropriate and subsequently used. It should also be noted that the host cell and the vector are advantageously used in such a combination that they are mutually functional to be capable of expressing DNA coding for the peptide of interest. Examples of the preferred vector-host combination include the combination of COS cell or CHO cell with a vector containing the early promoter of simian virus 40 (SV40), a vector containing EF-1 α promoter (EF promoter) or a vector containing SR α promoter and so forth, the combination of yeast *Saccharomyces cerevisiae* with a vector containing the promoter of a 3-phosphoglycerate kinase gene and so forth, as well as the combination of *E. coli* HB101 with a vector containing tryptophan promoter derived *E. Coli*.

[0046] The host transformed with the expression vector can be cultured using nutrient media in accordance with known general methods for culturing microorganisms, animal cells or insect cells. The peptide of interest produced by the transformed host can be purified, isolated and recovered from the culture broth by making reference to many articles and literature (e.g., "Shin Seikagaku Jikken Koza 1. Tanpakushitsu I", edited by The Japanese Biochemical Society, published by Tokyo Kagaku Dojin, 1990)]. Briefly, the peptide of interest can be obtained in a pure form using at least one method selected from among desalting, concentration, salting out, ultrafiltration, ion-exchange chromatography, reverse-phase chromatography, isoelectric chromatography, affinity chromatography, and gel filtration.

[0047] A peptide having biological activity can be obtained by the same procedures as those described above for obtaining the peptide having affinity for a specified phospholipid. Briefly, human TM, for instance, can be obtained by chemical synthesis or using genetic engineering or by the combination of both.

[0048] A chemical substance exhibiting a pharmacological action can be obtained by chemical synthesis. If it is difficult to obtain by chemical synthesis or if the cost for chemical synthesis is exorbitant, it may be extracted, separated or purified from natural products or it may be separated and purified from the supernatant of the culture broth of a microorganism.

[0049] If the drug of the present invention is such that both the biologically active substance and the substance having affinity for a phospholipid are peptides and that the N terminus of either one of the peptides and the C terminus of the other are joined linearly by a peptide bond, with a peptide of a given length being optionally interposed as a linker, the drug can be directly obtained by a process characterized in that at least one of the following steps is performed:

- a) the step of obtaining a peptide having the amino acid sequence of the drug of interest by chemical synthesis;
- b) the step of obtaining DNA having a sequence coding for the amino acid sequence of the drug of interest;
- c) the step of incorporating said DNA into a vector so as to give a replicable recombinant DNA containing said DNA;
- d) the step of transforming a host cell with said recombinant DNA to give a transformant capable of expressing the peptide of interest;
- e) the step of culturing said transformant to produce the peptide of interest and recovering said peptide from the culture broth.

[0050] The peptide having the amino acid sequence of the drug of interest can be obtained by chemical synthesis, typically using an automatic peptide synthesizer.

[0051] The DNA having a sequence coding for the amino acid sequence of the drug of interest may typically be prepared in the following manner. Unless otherwise expressly stated, general genetic engineering techniques can be implemented on the basis of the procedures described in literature [such as "Molecular Cloning, A LABORATORY MANUAL", Second Edition, T. Maniatis et al., Cold Spring Harbor Laboratory Press (1989)]. To begin with, cDNA prepared on the basis of mRNA extracted from human cells or organs, a commercially available human cDNA library or human chromosomal DNA is used as template DNA. Then, by referring to the known DNA sequence of a peptide hav-

ing biological activity (e.g. human TM DNA), the template DNA is screened using a DNA probe chemically synthesized with an automatic DNA synthesizer to obtain DNA (I) that codes for part or all of the peptide having biological activity. DNA (I) can also be obtained by chemical synthesis alone using an automatic DNA synthesizer. Similarly, DNA (II) can be produced that codes for a peptide having affinity for a specified phospholipid (e.g. the C-terminal region of human factor VIII). Another preferred method for obtaining DNA (I) and DNA (II) is one utilizing PCR. Briefly, by referring to the known DNA sequence (e.g., human TM DNA or human factor VIII DNA), a DNA primer is chemically synthesized with optional sequences and restriction enzyme recognizing sites being attached as required, and PCR is performed using the above-mentioned cDNA as a template DNA such that the desired DNA is obtained. It should be noted that PCR can be performed by making reference to literature [e.g., "PCR Protocols, A Guide to Methods and Applications", Michael A.I. et al., Academic Press (1990)]. The thus obtained DNA (I) and DNA (II) are optionally digested with restriction enzymes and bound together, with a chemically synthesized DNA linker being optionally interposed, thereby yielding a DNA fragment containing the DNA coding for the drug of the present invention.

[0052] The step of incorporating the stated DNA into a vector so as to give a replicable recombinant DNA containing said DNA, the step of transforming a host cell with said recombinant DNA to give a transformant capable of expressing the drug of interest, and the step of culturing said transformant to produce the drug of interest and recovering said drug from the culture broth can be implemented by the same procedures as those described above for obtaining the peptide having affinity for a specified phospholipid.

[0053] If the novel peptide having affinity for phosphatidylserine according to the present invention is bound to a biologically active substance or its aggregate or encapsulation, there is provided a means of enabling the biologically active substance or its aggregate or encapsulation to be delivered selectively on the surface of cells that are not normal, as exemplified by damaged, denatured or activated cells. Take, for example, the case where the biologically active substance is contained within encapsulations, the substance having affinity for a specified phospholipid is modified with a substance having affinity for the components making up the skeleton of the encapsulations and thereafter mixed with the encapsulations containing the biologically active substance, whereby said substance having affinity for a specified phospholipid is bound to the surfaces of the encapsulations to yield the drug of the present invention. To give a preferred example for the case where the biologically active substance is contained within liposomes, the substance having affinity for a specified phospholipid is modified with a suitable phospholipid such as phosphatidylethanolamine and thereafter mixed with the liposomes, whereby the substance having affinity for a specified phospholipid is bound to the surface layer of each liposome to yield the drug of the present invention.

[0054] The substances or drug of the present invention, for example, the substances to be described in the Examples were not found to have any significant toxicity.

[0055] In addition, the drug of the present invention may appropriately be combined with pharmaceutical carriers and media such as sterilized water, biological saline, vegetable oils, mineral oils, higher alcohols, higher fatty acids and innocuous organic solvents, etc. and further with optional excipients, coloring agents, emulsifiers, suspending agents, surfactants, solubilizers, anti-adsorbents, stabilizers, preservatives, humectants, antioxidants, buffering agents, isotonic solutions, palliatives, etc. so as to take the form of pharmaceutical compositions (e.g., injections and oral drugs) or kits. The drug of the present invention can be administered systemically or topically and either rapidly or in a sustained manner, preferably by peroral routes, for example, by intravenous injection, intracoronary injection, intramuscular injection, intraperitoneal injection or subcutaneous injection, etc. However, the use of the drug of the present invention is by no means limited to these methods of administration. In addition, it may be used in combination with other drugs.

[0056] The dose of administration of the drug of the present invention can be adjusted as appropriate for the biologically active substance contained in said drug and depending upon the severity of the disease the patient is suffering from.

[0057] The present invention also provides a novel method for delivery of the biologically active substance in a site-selective manner so as to enhance its action and efficacy by a marked degree. Stated more specifically, the present invention provides a method by which the substance having affinity for a specified phospholipid is bound to the desired biologically active substance so that the latter is delivered on the surface layer of cells to exhibit an enhanced action and efficacy. Herein, the phospholipid which serves as targeted molecules to deliver the biologically active substance consists of molecules that compose cell membranes which are possessed by all cells without exception and, in this respect, it is totally different from the aforementioned specific antigens as the specific receptors, molecules and so forth which are composed of polypeptides. Therefore, all cells, as well as all tissues and organs that are composed of cells can potentially provide sites where the activity of the biologically active substance in the drug of the present invention will increase. In addition, if the affinity conferred on the biologically active substance is for a specified phospholipid, the activity of the biologically active substance can be selectively increased in certain of the cells and in certain of the tissues and organs that are composed of the cells. Thus, the present invention provides a drug delivery method or system based on the entirely new concept that the action and efficacy of a biologically active substance is enhanced by its selective delivery on the surface layers of cells, tissues and organs that are not normal. As will be described in the Examples, the present inventors prepared substances having affinity for a phospholipid, in which a peptide having affin-

ity for a phospholipid was bound to TM, UTI, the second region of UTI or MCP. As it turned out, the TM having affinity for a phospholipid, the UTI having affinity for a phospholipid and the second region of UTI having affinity for a phospholipid were sufficiently increased in their activity and ability of being localized on phospholipid to exhibit an enhanced action and efficacy. The increase in activity and ability of being localized on phospholipid can also be verified for the MCP having affinity for a phospholipid by measuring its action in suppressing complement-dependent hemolysis as described in a literature ("Hotaigaku", Inai M. et al., published by Ishiyaku Shuppan Kabushiki Kaisha, 1982). The Examples provide illustrations of a drug delivery method or system that are based on the aforementioned entirely new concept that the action and efficacy of a biologically active substance is enhanced by selective aggregation of said substance on the surface layers of cells, tissues and organs at various sites such as a site where a blood coagulation is in progress, a site where the so-called immune response reactions of cells such as their activation and impairment due to inflammation or immunocytes are in progress, and a site impaired by active oxygen, and a site where a cell activation and impairing reaction is in progress due to active proteases. In summary, the present invention provides a drug and a novel peptide that are useful as preventives and therapeutics of diseases involving coagulopathy, inflammations and immune response reactions, as well as DNA necessary for producing them and a process for producing said drug. It should be noted that the drug of the present invention is by no means limited to pharmaceuticals and may be used as clinical or research reagents and the like.

[0058] The present invention will now be described below more specifically by means of working examples, which are given herein for the mere purpose of illustrating the practice of the invention and are in no way intended to limit the same. The abbreviations used in the following description are based on those which are conventional in the art concerned.

[0059] Unless otherwise noted, genetic engineering technology was implemented adopting the protocols described in books such as "Molecular Cloning, A LABORATORY MANUAL", Second Edition, T. Maniatis et al., Cold Spring Harbor Laboratory Press (1989), "A Practical Guide to Molecular Cloning", 2nd Edition, Bernard Perb et al., John Wiley & Sons (1988), "PCR Protocols, A Guide to Methods and Applications", Michael A.I. et al., Academic Press (1990), "Shin Saibo Kogaku Jikken Purotokol", edited by Department of Oncology, Institute of Medical Science, University of Tokyo, published by Shujunsha, 1991, and "Idenshi Kogaku Handobukku", edited by Muramatsu M. et al., published by Yodoshinsha, 1991, as well as the protocols attached to the reagents or equipment used.

[0060] The *E. coli* strains bearing pM1354 and pM1357, respectively, which were expression plasmids for the rsTMTd and rsTMC2 disclosed in the Examples were deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, JAPAN under date of December 16, 1996 (under respective Accession Numbers P-16008 and P-16009) and a transfer from the original deposit to an international deposit was effected under date of December 8, 1997 (under respective Accession Numbers FERM BP-6194 and FERM BP-6195). The *E. coli* strain bearing pM851 which was an expression plasmid for human MCP was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, JAPAN under date of January 22, 1992 (under Accession Number 2-12715) and a transfer from the original deposit to an international deposit was effected under date of February 18, 1993 (under Accession Number FERM BP-4195).

Example 1

Chemical Synthesis of Peptides Having Affinity for Phosphatidylserine

[0061] Peptides having the amino acid sequence of Sequence ID Nos. 5, 6 and 22 were synthesized by a solid-phase method using an automatic peptide synthesizer (Model 432A, product of Applied Biosystems). Unless otherwise expressly stated, the operating methods were in accordance with the attached operating manual. Following cleavage, deprotection and precipitation in ether, peptide were obtained by removing the ether and the resulting residue was freeze-dried. The freeze-dried peptides were dissolved in 10% acetonitrile with 0.1% trifluoroacetic acid, and using a C18 column (CAPCELLPACC18AG120, product of Shiseido Co., Ltd.) and high-performance liquid chromatography (625LC System, product of Waters), the peptides were purified by a 10% - 60% acetonitrile with 0.1% trifluoroacetic acid linear density gradient.

[0062] The yields of the respective peptides were 10 mg, 6 mg and 12 mg.

Example 2

Cloning of Human TM cDNA and Preparation of Human TM Expressing Plasmids

[0063] Total RNA was isolated from about 20 g of human placenta by a guanidium isothiocyanate method. A portion (10 mg) of the resulting RNA was passed twice through a oligo(dT)-cellulose column (type 7, product of Pharmacia) to

recover about 90 µg of poly(A)⁺ RNA. Then, using the resulting poly(A)⁺ RNA as a starting material, single-stranded cDNA was synthesized. Briefly, single-stranded cDNA was synthesized by means of a reverse transcriptase using 10 µg of the poly(A)⁺ RNA as a template and an oligo(dT) as a primer in the usual manner.

[0064] In a separate step, by referring to a known sequence of human TM DNA [K. Suzuki et al., EMBO J., Vol. 6, 1891 (1987) and R.W. Jackman et al., Proc. Natl. Acad. Sci. USA, Vol. 84, 6425 (1987)], six DNA primers (S1 - S3 and A1 - A3; see Figs. 1 and 2), each corresponding to a portion of the DNA sequence of the human TM gene and containing a suitable restriction enzyme recognition site at 5' end, were synthesized with a chemical synthesizer (Model 381, product of Applied Biosystems). As for S3, a restriction enzyme XhoI recognition site was introduced by silent mutation. Speaking of A3, it contained a DNA sequence corresponding to a termination codon. The synthesized DNA primers were purified on an OPC column (product of Applied Biosystems).

[0065] Then, using the aforementioned single-stranded cDNA as template DNA, PCR was performed using the chemically synthesized DNA primers in accordance with the reaction solution's recipe shown in Table 1, and the human TM cDNA was amplified in three divided portions (for the correspondence between the DNA primers used and the DNA to be amplified, see Table 2). DNA amplification was performed with a thermal cycler (Model PJ1000, product of Perkin-Elmer Cetus) by repeating 30 reaction cycles, each consisting of 94°C × 1 min, 55°C × 2 min and 72°C × 3 min; after the end of the reaction, a portion of the reaction solution was sampled and subjected to agarose gel electrophoresis, which verified the amplification of the intended sizes of DNA.

Table 1

PCR Reaction Solution's Recipe	
Distilled water	77 µL
Buffer*)	10 µL
dNTPs mixed solution (2.5 mM)	8 µL
Sense primer (1 µg/µL)	1 µL
Antisense primer (1 µg/µL)	1 µL
Template DNA (ca. 20 ng/µL)	2.5 µL
Heat-resistant DNA polymerase (5 units/µL)	0.5 µL
Total	100 µL

*) Buffer: 0.1 M Tris-HCl (pH = 8.3)
0.5 M KCl
15 mM MgCl₂

Table 2

Combinations of Sense and Antisense Primers vs Amplified Genes			
	Sense primer	Antisense primer	Amplified DNA
Combination I	S1	A1	Fragment I
Combination II	S2	A2	Fragment II
Combination III	S3	A3	Fragment III

[0066] The amplified DNA (Fragments I - III) were purified and recovered from the reaction solution by a phenol/chloroform treatment and ethanol precipitation. Fragments I, II and III were digested with restriction enzyme pairs SalI/BamHI, HindIII/SalI and PstI/BamHI, respectively, and thereafter subcloned into a cloning vector pUC118 in the usual manner to give pUC118-FI, pUC118-FII and pUC118-FIII. Subsequently, pUC118-FI was digested with a restriction enzyme pair HindIII/DdeI, pUC118-FII with DdeI/SalI and pUC118-FIII with XhoI/EcoRI and subjected to agarose gel electrophoresis in the usual manner, whereby DNA fragments of Ca. 450 bp, ca. 650 bp and ca. 650 bp were separated and recovered. The three recovered DNA fragments and the product of digestion of pUC118 with a restriction

enzyme pair HindIII/EcoRI were ligated in the usual manner to prepare a plasmid pUC118-TM bearing a full length of human TM cDNA containing a signal peptide (the process of construction of the plasmid is shown in Fig. 3). In the usual manner, the sequence determination of the cDNA obtained was carried out with a DNA sequencer (370A, product of Applied Biosystems), whereupon it was verified to be human TM cDNA.

[0067] In the next step, pUC118-TM was digested with restriction enzymes Sall and EcoRI and subjected to agarose gel electrophoresis in the usual manner, thereby separating a DNA fragment in pure form having a length of about 1.7 kbp. The fragment was inserted between cloning sites, PstI and EcoRI, in a mammalian cell expression vector pcDL-SR α 296 [Y. Takebe et al., Mol. Cell. Biol., Vol. 8, 466 (1988)] together with PstI-Sall linker (5'-TCGATGCA-3') that had been synthesized with a chemical synthesizer (supra) and purified on an OPC column (supra), thereby constructing a human TM expressing vector pSR α -TM (the process of its construction is shown in Fig. 3).

Example 3

Preparation of Plasmids Expressing Soluble Human TM Having Affinity for Phosphatidylserine

(1) Preparing pM1350

[0068] Four species of single-stranded DNA (F1 - F4; see Fig. 4) were synthesized with a chemical synthesizer (supra) and F1 and F2 were annealed in the usual manner and so were F3 and F4, thereby yielding DNA fragment A which was ca. 30 bp in length and which had a BsmI cleaved surface at 5' end and an EcoRI cleaved surface at 3' end, as well as DNA fragment B which was ca. 90 bp in length and which had an NspV cleaved surface at 5' end and an EcoRI cleaved surface at 3' end. Then, the pUC118-TM prepared in Example 2 was digested with restriction enzymes BsmI and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.6 kbp. This DNA fragment was ligated with fragment A in the usual manner to yield pUC118-TM sub1. Subsequently, the pUC118-TM sub1 was digested with restriction enzymes MluI and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 0.8 kbp. In a separate step, the pSR α -TM prepared in Example 2 was digested with restriction enzymes MluI and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.4 kbp. The two DNA fragments of ca. 0.8 kbp and ca. 4.4 kbp in length were ligated in the usual manner to yield pSR α -TM sub1. Further, the pSR α -TM sub1 was digested with restriction enzymes NspV and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 5.2 kbp. This DNA fragment was ligated with fragment B in the usual manner to yield pM1350 (the process of its construction is shown in Fig. 6). This plasmid contained DNA consisting of a nucleotide sequence in which the nucleotide sequence of Sequence ID No. 16 was linked to the 3' side of the nucleotide sequence of Sequence ID No. 21; using this plasmid, one can produce soluble human TM having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the amino acid sequence of Sequence ID No. 5 is linked to the C terminus of the amino acid sequence of Sequence ID No. 4 (said soluble human TM is hereinafter designated as rsTMVIII).

(2) Preparing pM1357

[0069] By referring to a known DNA sequence of human factor VIII [William I. Wood et al., Nature, Vol. 312, 330 (1984)], a total of four DNA primers, two of them corresponding to a portion of the DNA sequence of the human factor VIII (S4 and A4; see Figs. 1 and 2) and two others (S5 and A5; see Figs. 1 and 2) which corresponded to another portion of the DNA sequence of the human factor VIII and which contained a suitable restriction enzyme recognition site at 5' end, were synthesized with a chemical synthesizer (supra).

[0070] It should be added that A5 contained a DNA sequence corresponding to a termination codon. The synthesized DNA primers were purified on an OPC column (supra).

[0071] Then, using the single-stranded cDNA synthesized using the human placental poly(A)⁺ RNA in Example 2 as template DNA, cDNA coding for a region including the C2 region of human factor VIII was amplified by PCR using chemically synthesized DNA primers S4 (as sense primer) and A4 (as antisense primer) by means of the same reaction solution as identified in Table 1. DNA amplification was performed with a thermal cycler (supra) by repeating 40 reaction cycles, each consisting of 94°C × 1 min, 55°C × 2 min and 72°C × 3 min; after the end of the reaction, a portion of the reaction solution was sampled and subjected to agarose gel electrophoresis, which at least verified the amplification of the intended size of DNA.

[0072] Subsequently, using the resulting DNA fragment produced by PCR as template DNA, PCR was performed using chemically synthesized DNA primers S5 (as sense primer) and A5 (as antisense primer) by means of the same reaction solution as identified in Table 1, thereby selectively amplifying cDNA coding for the C2 region of human factor VIII. DNA amplification was performed with the thermal cycler (supra) by repeating 30 reaction cycles, each consisting

of 94°C × 1 min, 55°C × 2 min and 72°C × 3 min; after the end of the reaction, a portion of the reaction solution was sampled and subjected to agarose gel electrophoresis, which verified the amplification of the intended size of DNA.

[0073] The amplified DNA was purified and recovered from the reaction solution by a phenol/chloroform treatment and ethanol precipitation; the recovered DNA was digested with restriction enzymes BsmI and EcoRI to yield a DNA fragment ca. 0.5 kbp in length which had a BsmI cleaved surface at 5' end and an EcoRI cleaved surface at 3' end. Then, the pUC118-TM prepared in Example 2 was digested with restriction enzymes BsmI and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.6 kbp. This DNA fragment was ligated with the previously obtained DNA fragment ca. 0.5 kbp in length in the usual manner to yield pUC118-TM sub2. Subsequently, the pUC118-TM sub2 was digested with restriction enzymes MluI and NspV and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 1.2 kbp. In a separate step, the pM1350 prepared in Example 3(1) was digested with restriction enzymes MluI and NspV and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.5 kbp. The two DNA fragments ca. 1.2 kbp and ca. 4.5 kbp in length were ligated in the usual manner to yield pM1357 (the process of its construction is shown in Fig. 7). This plasmid contained DNA consisting of a nucleotide sequence in which the nucleotide sequence of Sequence ID No. 20 was linked to the 3' side of the nucleotide sequence of Sequence ID No. 21; using this plasmid, one can produce soluble human TM having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the amino acid sequence of Sequence ID No. 9 is linked to the C terminus of the amino acid sequence of Sequence ID No. 4 (said soluble human TM is hereinafter designated as rsTMC2).

(3) Preparing pM1356

[0074] By referring to known DNA sequence of human factor VIII (supra), one DNA primer (S6, see Fig. 1) which corresponded to a portion of the DNA sequence of human factor VIII and which contained a suitable restriction enzyme recognition site at 5' end was synthesized with a chemical synthesizer (supra). The synthesized DNA primer was purified on an OPC column (supra).

[0075] Then, using the DNA fragment which coded for a region including the C2 region of human factor VIII that was amplified by PCR using DNA primers S4 and A4 in Example 3(2) as template DNA, PCR was performed with the same reaction solution as identified in Table 1 using chemically synthesized DNA primer S6 (as sense primer) and DNA primer A5 (as antisense primer) synthesized in Example 3(2), thereby selectively amplifying a DNA fragment coding for 60 amino acids at the C terminus of the C2 region of human factor VIII. DNA amplification was performed with a thermal cycler (supra) by repeating 30 reaction cycles, each consisting of 94°C × 1 min, 55°C × 2 min and 72°C × 3 min; after the end of the reaction, a portion of the reaction solution was sampled and subjected to agarose gel electrophoresis, which verified the amplification of the intended size of DNA.

[0076] The amplified DNA was purified and recovered from the reaction solution by a phenol/chloroform treatment and ethanol precipitation; the recovered gene was digested with restriction enzymes BsmI and EcoRI to yield a DNA fragment ca. 0.2 kbp in length which had a BsmI cleaved surface at 5' end and an EcoRI cleaved surface at 3' end. Then, the pUC118-TM prepared in Example 2 was digested with restriction enzymes BsmI and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.6 kbp. This DNA fragment was ligated with the previously prepared DNA fragment about 0.2 kbp in length in the usual manner to yield pUC118-TM sub3. Subsequently, the pUC118-TM sub3 was digested with restriction enzymes MluI and NspV and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 0.9 kbp. In a separate step, the pM1350 prepared in Example 3(1) was digested with restriction enzymes MluI and NspV and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of Ca. 4.5 kbp. The two DNA fragments of Ca. 0.9 kbp and Ca. 4.5 kbp in length were ligated in the usual manner to yield pM1356 (the process of its construction is shown in Fig. 8). This plasmid contained DNA consisting of a nucleotide sequence in which the nucleotide sequence of Sequence ID No. 19 was linked to the 3' side of the nucleotide sequence of Sequence ID No. 21; using this plasmid, one can produce soluble human TM having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the amino acid sequence of Sequence ID No. 8 is linked to the C terminus of the amino acid sequence of Sequence ID No. 4 (said soluble human TM is hereinafter designated as rsTMC2D).

(4) Preparing pM1354

[0077] Four species of single-stranded DNA (F5 - F8; see Fig. 4) were synthesized with a chemical synthesizer (supra) and F5 and F6 were annealed in the usual manner and so were F7 and F8, thereby yielding DNA fragment C which was ca. 60 bp in length and which had an NspV cleaved surface at 5' end and an EcoRI cleaved surface at 3' end, as well as DNA fragment D which was ca. 90 bp in length and which had an NspV cleaved surface at 5' end and an EcoRI cleaved surface at 3' end. Then, the pSRα-TM sub1 prepared in Example 3(1) was digested with restriction enzymes NspV and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA

fragment of Ca. 5.2 kbp. This fragment was ligated with fragment C in the usual manner to yield pSR α -TM sub2. Subsequently, the pSR α -TM sub2 was digested with restriction enzymes NspV and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 5.3 kbp. This fragment was ligated with fragment D in the usual manner to yield pM1354 (the process of its construction is shown in Fig. 9). This plasmid contained DNA consisting of a nucleotide sequence in which the nucleotide sequence of Sequence ID No. 17 was linked to the 3' side of the nucleotide sequence of Sequence ID No. 21; using this plasmid, one can produce soluble human TM having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the amino acid sequence of Sequence ID No. 6 is linked to the C terminus of the amino acid sequence of Sequence ID No. 4 (said soluble human TM is hereinafter designated as rsTMTd).

(5) Preparing pM1355

[0078] The pSR α -TM sub2 prepared in Example 3(4) was digested with restriction enzymes NspV and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 5.3 kbp. This DNA fragment and fragment C prepared in Example 3(4) were ligated in the usual manner to thereby yield pSR α -TM sub3. Subsequently, the pSR α -TM sub3 was digested with restriction enzymes NspV and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of about 5.4 kbp. This fragment and the fragment D prepared in Example 3(4) were ligated in the usual manner to yield pM1355 (the process of its construction is shown in Fig. 10). This plasmid contained DNA consisting of a nucleotide sequence in which the nucleotide sequence of Sequence ID No. 18 was linked to the 3' side of the nucleotide sequence of Sequence ID No. 21; using this plasmid, one can produce soluble human TM having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the amino acid sequence of Sequence ID No. 7 is linked to the C terminus of the amino acid sequence of Sequence ID No. 4 (said soluble human TM is hereinafter designated as rsTMTd-3).

(6) Preparing pM1358

[0079] Two species of single-stranded DNA (F9 and F10; see Fig. 4) were synthesized with a chemical synthesizer (supra) and F9 and F10 were annealed in the usual manner to yield DNA fragment E ca. 60 bp in length which had an NspV cleaved surface at 5' end and an EcoRI cleaved surface at 3' end. Then, the pM1354 prepared in Example 3(4) was digested with restriction enzymes NspV and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of Ca. 5.3 kbp. This fragment was ligated with fragment E in the usual manner to yield pM1358 (the process of its construction is shown in Fig. 14). This plasmid contained DNA consisting of a nucleotide sequence in which the nucleotide sequence of Sequence ID No. 26 was linked to the 3' side of the nucleotide sequence of Sequence ID No. 21; using this plasmid, one can produce soluble human TM having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the amino acid sequence of Sequence ID No. 22 is linked to the C terminus of the amino acid sequence of Sequence ID No. 4 (said soluble human TM is hereinafter designated as rsTMTdD).

Example 4

Preparing Unmodified Soluble TM Expressing plasmid

[0080] Two species of single-stranded DNA (F11 and F12; see Fig. 5) were synthesized with a chemical synthesizer (supra) and both were annealed in the usual manner to yield DNA fragment F ca. 20 bp in length which had a BsmI cleaved surface at 5' end and an EcoRI cleaved surface at 3' end. Then, the pUC118-TM prepared in Example 2 was digested with restriction enzymes BsmI and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.6 kbp. This fragment was ligated with fragment F in the usual manner to yield pUC118-sTM. Subsequently, the pUC118-sTM was digested with restriction enzymes MluI and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 0.8 kbp. In a separate step, the pSR α -TM prepared in Example 2 was digested with restriction enzymes MluI and EcoRI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.4 kbp. The two DNA fragments of ca. 0.8 kbp and ca. 4.4 kbp in length were ligated in the usual manner to yield pM1399. This plasmid contained DNA having the base sequence of Sequence ID No. 21; using this plasmid, one can produce unmodified soluble human TM of Sequence ID No. 4 (which is hereinafter designated as rsTM).

Example 5

Expressing rsTMVIII, rsTMC2, rsTMC2D, rsTMTd, rsTMTd-3, rsTMTdD and rsTM

5 **[0081]** Each of the plasmids prepared in Examples 3 and 4, i.e., pM1350, pM1357, pM1356, pM1354, pM1355, pM1358 and pM1399, was transfected into COS-1 cells (ATCC CRL-1650) by a DEAE dextran method [adapted from the method described in Lauren M. Sompayrac et al., Proc. Natl. Acad. Sci. USA, Vol. 78, 7575 (1981)], thereby expressing soluble human TM having affinity for phosphatidylserine and unmodified soluble human TM. Stated more specifically, Ca. 3×10^5 cells/9 cm² were inoculated in plastic tissue culture plate and cultivated at 37°C for one day in 10 2 mL of a Dulbecco modified Eagle's medium (hereinafter abbreviated as DMEM) containing 10% fetal calf serum. After washing three times with 2 mL of DMEM, culture medium was replaced by 0.7 mL of DMEM containing 1 µg of each of the plasmids, 50 mM Tris-HCl (pH 7.4), 0.2 mg/mL of DEAE dextran and 150 µM chloroquine. After cultivation at 37°C for 4 h, the culture solution was removed by suction; after washing once with 2 mL of DMEM and once with DMEM containing 10% fetal calf serum, 2 mL of DMEM containing 10% fetal calf serum was added and cultivation was continued 15 at 37°C for 24 h. Thereafter, the culture medium was replaced by DMEM containing 0.1% BSA and cultivation was continued at 37°C for an additional 72 h, and the supernatant of the culture solution was collected. It was found that collected culture solution contained various types of soluble human TM having affinity for phosphatidylserine (rsTMVIII, rsTMC2, rsTMC2D, rsTMTd, rsTMTd-3 and rsTMTdD) and unmodified human soluble TM (rsTM) in amounts of 1 - 5 µg/mL.

20 **[0082]** The collected culture solution was desalted and concentrated using an ultrafiltration membrane having a molecular weight cutoff value of 3×10^4 . After pH was adjusted to 7.5, the concentrate was passed through a DIP-thrombin-agarose column preconditioned with 0.02 M Tris-HCl buffer containing 0.1 M NaCl, 1 mM benzamidine hydrochloride, 0.5 mM CaCl₂ and 0.5% Triton X-100, whereby active fractions were adsorbed on the column. Then, after washing with the same buffer as used for preconditioning, the active fractions were eluted by a 0.1 - 1 M NaCl linear density gradient using 0.02 M Tris-HCl buffer containing 0.1 mM EDTA, 1 mM benzamidine hydrochloride and 0.5% Triton X-100; thus, various types of soluble human TM having affinity for phosphatidylserine (i.e., rsTMVIII, rsTMC2, 25 rsTMC2D, rsTMTd, rsTMTd-3 and rsTMTdD) and unmodified soluble human TM (rsTM) were obtained in pure form.

Example 6

30 Preparing Liposomes

[0083] Liposomes were prepared from different phospholipids in accordance with the method of Kaneda, Y [Jikken Igaku, Vol. 12, 184 (1994)]. Stated more specifically, 10 mg of phosphatidylserine (derived from bovine brain and product of Sigma), 10 mg of phosphatidylcholine (derived from egg yolk and product of Sigma), 10 mg of phosphatidylethanolamine (from bovine brain and product of Sigma) or 10 mg of phosphatidic acid (derived from egg yolk and product of Sigma), each being dissolved in chloroform, was charged into an egg plant type flask either individually or in admixture. After drying under nitrogen gas, 50 µL of 10 mM potassium phosphate solution and 450 µL of tetrahydrofuran were added and the phospholipid was redissolved in the same egg plant type flask. The flask was fitted on a rotary evaporator 40 (Model REN-1, product of Iwaki Glass Co., Ltd.) and dried under the vacuum as it was whirled in a water bath at 45°C, whereupon thin phospholipid films formed on the inner glass surface of the flask. Following the addition of 200 µL of 30 mM Tris-imidazole buffer (pH 8.4) containing 20 mM CaCl₂ and 0.22 M NaCl (the buffer is hereinafter abbreviated as TIBS), the flask was shaken vigorously for 30 seconds with a vortex mixer and left to stand at 37°C in a constant temperature bath for 30 seconds; the process of vigorous shaking and subsequent standing was repeated 8 times. The 45 flask was then placed in a water tank in an ultrasonic cleaner (Model UT-53, product of Sharp Corp.) and sonicated for 5 seconds; thereafter, the flask was shaken vigorously for 30 seconds with the vortex mixer and 300 µL of TIBS (supra) was added, followed by 30-min shaking in a constant temperature bath equipped with a shaker to prepare a liposome solution. The solution contained 20 mg/mL of a phospholipid of interest in the form of liposomes.

50 Example 7

Measuring the Ability to Accelerate the Activation of Protein C

[0084] Measurements of the ability to accelerate the activation of protein C in the absence of phospholipids were conducted in accordance with the method of Takahashi et al. [Thrombosis and Haemostasis, Vol. 73, 805 (1995)]. Stated more specifically, 75 µL of TIBS (supra) and 25 µL of TIBS containing 40 U/mL of bovine thrombin (product of Mochida Pharmaceutical Co., Ltd.) were mixed either with 25 µL of the supernatant of the culture of COS-1 cells in which each of the soluble human TM species having affinity for phosphatidylserine which are described in Example 5 was

expressed, optionally after dilution with the supernatant of the culture of COS-1 cells, or with 25 μ L of the supernatant of the culture of COS-1 cells in which unmodified soluble human TM was expressed, optionally after dilution with the supernatant of the culture of COS-1 cells, and the mixture was heated at 37°C for 10 minutes. Then, 25 μ L of TIBS containing 12 U/mL of human protein C (product of American Diagnostica) was added and reaction was performed at 37°C for 10 minutes followed by the addition of 100 μ L of TIBS containing 0.15 U/mL of antithrombin III (product of The Green Cross Corp.) and 15 U/mL of heparin (product of Mochida Pharmaceutical Co., Ltd.) to terminate the protein C activation reaction. The termination reaction was continued at 37°C for 10 minutes; thereafter, 250 μ L of TIBS containing 3.2 mM of synthetic substrate S-2366 (product of Daiichi Pure Chemicals Co., Ltd.) was added and reaction was performed at 37°C for 5 minutes. Subsequently, 1.5 mL of 50% aqueous acetic acid was added so that all reactions would terminate and the concentration of the synthetic substrate cleaved with the activated protein C was measured with a spectrophotometer (DU640, product of Beckman) at a wavelength of 405 nm.

[0085] The above-described method was partially modified to measure the ability to accelerate the activation of protein C in the presence of a phospholipid. Stated more specifically, 75 μ L of TIBS (supra) containing 6 mg/mL of the phosphatidylserine liposomes prepared in Example 6 and 2% BSA was heated at 37°C for 1 h in order to block the non-specific adsorption of polypeptides on the liposomes. Then, 25 μ L of the supernatant of the culture of COS-1 cells in which each of the soluble human TM species having affinity for phosphatidylserine which are described in Example 5 was expressed in such an amount as to provide the same value of action in accelerating the activation of protein C as measured in the absence of phospholipids by the above-described method was added, optionally after dilution with the supernatant of the culture of COS-1 cells or, alternatively, 25 μ L of the supernatant of the culture of COS-1 cells in which unmodified soluble human TM was expressed was added, optionally after dilution with the supernatant of the culture of COS-1 cells, and the mixture was subjected to reaction at 37°C for 2 h; subsequently, 25 μ L of TIBS containing 40 U/mL of bovine thrombin was added and reaction was performed at 37°C for 10 minutes. Then, 25 μ L of TIBS containing 12 U/mL of human protein C was added and reaction was performed at 37°C for 10 minutes; in the subsequent stage, the same procedure as in the above-described method was followed to measure the ability to accelerate the activation of protein C. It should be noted that the concentration of the synthetic substrate cleaved with the activated protein C was measured on the reaction solution from which the liposomes had been removed by centrifugation at 40,000 rpm for 15 minutes. The result is shown in Fig. 11, from which it was clear that the soluble human TM species having affinity for phosphatidylserine according to the present invention were significantly enhanced in their ability to accelerate the activation of protein C in the presence of phospholipids; the degree of improvement over rsTM was by a factor of about 5 in rsTMVIII, a factor of about 10 in rsTMC2D, factors of about 16 - 18 in rsTMTd, rsTMTdD and rsTMTd-3 and a factor of about 50 in rsTMC2. (The magnitude of the ability to accelerate the activation of protein C in the presence of the phosphatidylserine liposomes was indicated in terms of relative values, with the magnitude of the action in accelerating the activation of protein C in the absence of liposomes being taken as unity.)

Example 8

Specificity for A Specified Phospholipid (1)

[0086] In Example 6, liposomes were also prepared from phospholipids other than phosphatidylserine. Using those liposomes, a soluble human TM species having affinity for phosphatidylserine (rsTMC2) and the unmodified soluble human TM (rsTM) were measured for their ability to accelerate the activation of protein C in accordance with the methods described in Example 7. The result is shown in Fig. 12, from which it was clear that the ability to accelerate the activation of protein C of the soluble human TM of the present invention having affinity for phosphatidylserine was selective in that it increased markedly only in the presence of the phosphatidylserine liposomes. (The magnitude of the action in accelerating the activation of protein C in the presence of different kinds of liposomes was indicated in terms of relative values, with the magnitude of the action in accelerating the activation of protein C in the absence of liposomes being taken as unity.)

Example 9

Evaluation of the Ability to Bind to Phospholipids

[0087] A 96-well microtiter plate (Immulon I, product of Dynatec) was treated with 100 μ L of ethanol having either phosphatidylserine or phosphatidylcholine dissolved therein; the plate was air-dried to make a solid phase of each phospholipid in an amount of 1 μ g per well. Then, there was added 200 μ L of 10 mM Tris-HCl buffer (pH 7.4) containing 1% BSA and 0.15 M NaCl, and blocking was performed at 37°C for 2 h. After removing the blocking solution, 100 μ L of the supernatant of the culture of COS-1 cells that contained genetically expressed rsTMC2 (see Example 5) to which 20% aqueous BSA was added to provide a final BSA concentration of 1% were added, or 100 μ L of a similarly prepared

supernatant of the culture of COS-1 cells that contained either genetically expressed rsTMTd or rsTM and 1% BSA, and the mixture was subjected to reaction at 4°C for 16 h. After the end of the reaction, each of the phospholipid-bound soluble human TM species having affinity for phosphatidylserine and the unmodified soluble human TM were quantitated by enzyme immunoassay (EIA) in accordance with the common method described in a literature ["Koso Meneki Sokuteiho (3rd Ed.)", authored by Ishikawa E. et al., published by Igaku Shoin, 1987]. Stated more specifically, using an anti-human TM monoclonal antibody (24FM, product of Celbio) as a primary antibody and a horseradish peroxidase labelled anti-mouse IgG antibody (P0260, product of Daco) as a secondary antibody, and with tetramethylbenzidine as a color forming substrate, absorbance were measured with a spectrophotometer (Model NJ-2100, product of Intermed) at a wavelength of 450 nm. The result is shown in Fig. 13, from which it is clear that the soluble human TM species having affinity for phosphatidylserine have ability to bind only to phosphatidylserine and that the intensity of the binding is correlated with the magnitude of the ability to accelerate the activation of protein C in the presence of phospholipids (see Fig. 11).

Example 10

Cloning of UTI cDNA and Preparing UTI Expressing Plasmids

[0088] By referring to a known DNA sequence coding for human α -microglobulin [Kaumeyer, J.F. et al., Nucl. Acids Res., Vol. 14, 7839 (1986)], a DNA primer (S7, see Fig. 1) that corresponded to a portion of the DNA sequence of a human α -microglobulin signal peptide and which contained a suitable restriction enzyme recognition site at 5' end was synthesized with a chemical synthesizer (supra). In addition, by referring to a known DNA sequence of human UTI [Kaumeyer, J.F. et al., Nucl. Acids Res., Vol. 14, 7839 (1986)], a DNA primer (A6, see Fig. 2) that corresponded to a portion of the DNA sequence of human UTI and which contained a suitable restriction enzyme recognition site at 5' end was synthesized with the chemical synthesizer (supra). It should be noted that A6 contained a DNA sequence corresponding to a termination codon. The synthesized DNA primers were purified on an OPC column (supra).

[0089] Then, using a commercially available human liver derived cDNA (product of CLONTECH) as template DNA, PCR was performed using chemically synthesized DNA primers S7 and A6 in accordance with the reaction solution's recipe shown in Table 1 (supra), thereby amplifying a human α -microglobulin cDNA and a human UTI cDNA flanking on its 3' side. DNA amplification was performed with a thermal cycler (supra) by repeating 40 reaction cycles, each consisting of 94°C × 45 sec, 60°C × 45 sec and 72°C × 2 min and 30 sec; after the end of the reaction, a portion of the reaction solution was sampled and subjected to agarose gel electrophoresis, which verified the amplification of the desired size of DNA.

[0090] The amplified DNA was purified and recovered from the reaction solution by a phenol/chloroform treatment and ethanol precipitation; the recovered DNA was digested with a restriction enzyme pair BamHI/HindIII and thereafter subcloned into a cloning vector pUC119 in the usual manner to yield pM1212 (the process of its construction is shown in Fig. 15). In addition, the base sequences of the cDNA portions were determined with a DNA sequencer (supra) to verify that they consisted of a human α -microglobulin cDNA and a human UTI cDNA flanking on its 3' side.

[0091] Then, a DNA primer (S8, see Fig. 1) that contained a portion of a known DNA sequence of human α -microglobulin signal peptide and a portion of the known DNA sequence of human UTI and which also contained a suitable restriction enzyme recognition site at 5' end, and a DNA primer (A7, see Fig. 2) that contained a portion of the known DNA sequence of human UTI and which also contained a suitable restriction enzyme recognition site at 5' end were synthesized with the chemical synthesizer (supra). It should be noted that A7 contained a DNA sequence corresponding to a termination codon. The synthesized DNA primers were purified on the OPC column (supra).

[0092] Subsequently, using the thus prepared DNA primers, PCR was performed using the aforementioned pM1212 as template DNA in accordance with the reaction solution's recipe shown in Table 1 (supra), thereby amplifying human UTI cDNA having a human α -microglobulin signal peptide. DNA amplification was performed with the thermal cycler (supra) by repeating 25 reaction cycles, each consisting of 94°C × 30 sec., 55°C × 30 sec, and 72°C × 1 min. After the end of the reaction, a portion of the reaction solution was sampled and subjected to agarose gel electrophoresis, which verified the amplification of the intended size of DNA.

[0093] The amplified DNA was purified and recovered from the reaction solution by a phenol/chloroform treatment and ethanol precipitation; the recovered DNA was digested with a restriction enzyme pair XbaI/NotI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment ca. 0.5 kbp in length in the usual manner. The fragment was inserted between cloning sites, XbaI and NotI, in pEF-BOS2A which was an improved expression vector of a known mammalian cell expression vector pEF-BOS [S. Mizushima et al., Nucleic Acids Res., Vol. 18, 5322 (1990)] and a human UTI expressing vector pM1213 was constructed (the process of its construction is shown in Fig. 15). The improved expression vector pEF-BOS2A has the promoter region of a human polypeptide chain extension factor 1 α and the poly(A)⁺ signal sequence of SV40. The plasmid pM1213 contains DNA having the DNA sequence of Sequence ID No. 28 and using this, one can produce unmodified UTI of Sequence ID No. 24 (which is hereinafter des-

ignated as rUTI).

Example 11

5 Preparing Plasmid Expressing UTI Having Affinity for Phosphatidylserine

[0094] Two species of single-stranded DNA (F13 and F14; see Fig. 5) were synthesized with a chemical synthesizer (supra) and F13 and F14 were annealed in the usual manner to yield DNA fragment G ca. 70 bp in length which had an EcoRI cleaved surface at 5' end and a HindIII cleaved surface at 3' end. Then, fragment G was subcloned into a cloning vector pUC119 between cloning sites EcoRI and HindIII, thereby yielding pUC119-Multi. Subsequently, the pUC119-Multi was digested with restriction enzymes Eco47III and NotI and subjected to agarose gel electrophoresis, thereby yielding a DNA fragment of ca. 3.2 kbp. In a separate step, pM1213 prepared in Example 10 was digested with restriction enzymes Eco47III and NotI and also subjected to agarose gel electrophoresis, thereby yielding a DNA fragment of ca. 0.5 kbp. The two fragments of ca. 3.2 kbp and 0.5 kbp in length were ligated in the usual manner to yield pUC119-UTI-sub1.

[0095] In a separate step, by referring to a known DNA sequence of human UTI (supra) and a known DNA sequence of human factor VIII (supra), a DNA primer (S9; see Fig. 1) corresponding to a portion of the DNA sequence of a human UTI and a portion of the DNA sequence of a human factor VIII, and a DNA primer (A8; see Fig. 2) that corresponded to a portion of the DNA sequence of the human factor VIII and which also contained a suitable restriction enzyme recognition site at 5' end were synthesized with the chemical synthesizer (supra). It should be noted that A8 contained a DNA sequence corresponding to a termination codon. The synthesized DNA primers were purified on an OPC column (supra).

[0096] Then, using those DNA primers, PCR was performed using the pM1357 synthesized in Example 3(2) as template DNA and in accordance with the reaction solution's recipe shown in Table 1 (supra), thereby amplifying a DNA fragment of ca. 0.6 kbp that contained the C2 region of human factor VIII and which had a region corresponding to a portion of the DNA sequence of the human UTI at 5' end. DNA amplification was performed using a thermal cycler (supra) by repeating 30 reaction cycles, each consisting of 94°C × 1 min, 55°C × 2 min and 72°C × 3 min; after the end of the reaction, a portion of the reaction solution was sampled and subjected to agarose gel electrophoresis, which verified the amplification of the intended size of DNA. The amplified DNA was purified and recovered from the reaction solution by a phenol/chloroform treatment and ethanol precipitation, and then, the recovered DNA was digested with restriction enzymes BsgI and NotI. In a separate step, the aforementioned pUC119-UTI-sub1 was digested with restriction enzymes BsgI and NotI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 3.1 kbp. This fragment and the previously digested DNA were ligated in the usual manner to yield pUC119-UTI-C2-sub1. Subsequently, the pUC119-UTI-C2-sub1 was digested with restriction enzymes Eco47III and NotI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 1.0 kbp. In a separate step, the pM1213 prepared in Example 10 was digested with restriction enzymes Eco47III and NotI, thereby separating and recovering a DNA fragment of ca. 4.0 kbp. The two fragments ca. 1.0 kbp and ca. 4.0 kbp in length were ligated in the usual manner to yield pM1380 (the process of its construction is shown in Fig. 16). This plasmid contained DNA consisting of a nucleotide sequence in which the DNA sequence of Sequence ID No. 20 was linked to the 3' side of the nucleotide sequence of Sequence ID No. 28; using this plasmid, one can produce human UTI having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the amino acid sequence of Sequence ID No. 9 is linked to the C terminus of the amino acid sequence of Sequence ID No. 24 (said human UTI is hereinafter referred to as rUTIC2).

45 Example 12

Expression and Purification of rUTI and rUTIC2

[0097] The plasmids pM1380 and pM1213 prepared in Examples 11 and 10 were each transfected into COS-1 cells (supra) by a DEAE dextran method (supra), thereby expressing human UTI having affinity for phosphatidylserine and unmodified human UTI. Stated more specifically, ca. 3×10^5 cells/9 cm² were inoculated in each plastic tissue culture plate and cultivated at 37°C for one day in 2 mL of a Dulbecco modified Eagle's medium (supra) containing 10% fetal bovine serum. After washing three times with 2 mL of DMEM, culture medium was replaced by 0.7 mL of DMEM containing 1 µg of each of the plasmids, 50 mM Tris-HCl (pH 7.4), 0.2 mg/mL of DEAE dextran and 150 µM chloroquine. After cultivation at 37°C for 4 h, the culture solution was removed by suction; after washing once with 2 mL of DMEM and once with DMEM containing 10% fetal bovine serum, 2 mL of DMEM containing 10% fetal bovine serum was added and cultivation was continued at 37°C for 24 h. Thereafter, the culture medium was replaced by DMEM containing 0.1% BSA and cultivation was continued at 37°C for an additional 72 h, and the supernatant of the culture was collected. It

was found that collected supernatant of the culture contained human UTI having affinity for phosphatidylserine (rUTIC2) and unmodified human UTI (rUTI) in amounts of 1 - 5 µg/mL.

[0098] The collected culture solution was desalted and concentrated using an ultrafiltration membrane having a molecular weight cutoff value of 10^4 . The concentrate was passed through an anti-UTI antibody-Sepharose column preliminarily equilibrated with 10 mM phosphate buffer (pH 7.5), whereby active fractions were adsorbed on the column. Then, after washing with 10 mM phosphate buffer (pH 7.5) containing 0.5 M NaCl, the active fractions were eluted with 0.1 M citric acid (pH 2.0) and 0.1 M citric acid containing 3 M potassium thiocyanate. The eluted active fractions were dialyzed against 10 mM phosphate buffer (pH 7.5) to obtain pure forms of human UTI having affinity for phosphatidylserine (rUTIC2) and unmodified human UTI (rUTI).

Example 13

Preparing Plasmid Expressing the Second Region of UTI

[0099] Two species of single-stranded DNA (F15 and F16; see Fig. 5) were synthesized with a chemical synthesizer (supra) and both were annealed in the usual manner to yield DNA fragment H ca. 70 bp in length which had an Eco47III cleaved surface at 5' end and an Apal cleaved surface at 3' end. Then, the pUC119-UTI-sub1 prepared in Example 11 was digested with restriction enzymes Eco47III and Apal and subjected to agarose gel electrophoresis, thereby yielding a DNA fragment of ca. 3.4 kbp. This fragment was ligated with fragment H in the usual manner to yield pUC119-R-020-sub1. Subsequently, the pUC119-R-020-sub1 was digested with restriction enzymes Eco47III and NotI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 0.2 kbp. In a separate step, the pM1213 prepared in Example 10 was digested with restriction enzymes Eco47III and NotI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.0 kbp. This fragment and the previously obtained fragment of ca. 0.2 kbp were ligated in the usual manner to yield pM1370 (the process of its construction is shown in Fig. 17). The plasmid pM1370 contains DNA having the sequence of Sequence ID No. 27 and using this, one can produce a polypeptide in which 11 amino acids (Ala-Val-Leu-Pro-Gln-Glu-Glu-Glu-Gly-Asp-Gly) is attached to the N terminus of the second region of unmodified human UTI represented by Sequence ID No. 23 (said human UTI is hereinafter referred to as Rr-020).

Example 14

Preparing Plasmid Expressing the Second Region of UTI Having Affinity for Phosphatidylserine

[0100] The pUC119-R-020-sub1 prepared in Example 13 was digested with restriction enzymes BsgI and NotI and subjected to agarose gel electrophoresis, thereby yielding a DNA fragment of ca. 3.3 kbp. In a separate step, the pUC119-UTI-C2-sub1 prepared in Example 11 was digested with restriction enzymes BsgI and NotI and subjected to agarose gel electrophoresis, thereby yielding a DNA fragment of Ca. 0.5 kbp. The two fragments ca. 3.3 kbp and ca. 0.5 kbp in length were ligated in the usual manner to yield pUC119-R-020-C2-sub1. Subsequently, the pUC119-R-020-C2-sub1 was digested with restriction enzymes Eco47III and NotI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 0.8 kbp. In a separate step, the pM1213 prepared in Example 10 was digested with restriction enzymes Eco47III and NotI and also subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.0 kbp. The two fragments ca. 0.8 kbp and ca. 4.0 kbp in length were ligated in the usual manner to yield pM1317 (the process of its construction is shown in Fig. 18). This plasmid contains DNA made up of a nucleotide sequence in which the nucleotide sequence of Sequence ID No. 20 is linked to the 3' side of the nucleotide sequence of Sequence ID No. 27; using this, one can produce the second region of human UTI having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the sequence of 11 amino acids (Ala-Val-Leu-Pro-Gln-Glu-Glu-Glu-Gly-Asp-Gly) is linked to the N terminus of the amino acid sequence of Sequence ID No. 23 whereas the amino acid sequence of Sequence ID No. 9 is linked to the C terminus (said second region of human UTI is hereinafter designated rR-020C2).

Example 15

Expression and Purification of rR-020 and rR-020C2

[0101] The plasmids pM1371 and pM1370 prepared in Examples 14 and 13 were each transfected into COS-1 cells (supra) by a DEAE dextran method (supra), thereby expressing the second region of human UTI having affinity for phosphatidylserine and the second region of unmodified human UTI. Stated more specifically, Ca. 3×10^5 cells/9 cm² were inoculated in each plastic tissue culture plate and cultivated at 37°C for one day in 2 mL of a Dulbecco modified

Eagle's medium (supra) containing 10% fetal bovine serum. After washing three times with 2 mL of DMEM, culture medium was replaced by 0.7 mL of DMEM containing 1 µg of each of the plasmids, 50 mM Tris-HCl (pH 7.4), 0.2 mg/mL of DEAE dextran and 150 µM chloroquine. After cultivation at 37°C for 4 h, the culture solution was removed by suction; after washing once with 2 mL of DMEM and once with DMEM containing 10% fetal bovine serum, 2 mL of DMEM containing 10% fetal bovine serum was added and cultivation was continued at 37°C for 24 h. Thereafter, the culture medium was replaced by DMEM containing 0.1% BSA and cultivation was continued at 37°C for an additional 72 h, and the supernatant of the culture was collected. It was found that the collected culture medium contained the secondary region of human UTI having affinity for phosphatidylserine (rR-020C2) and the second region of unmodified human UTI (rR-020) in amounts of 1 - 5 µg/mL.

[0102] The collected culture solution was desalted and concentrated using an ultrafiltration membrane having a molecular weight cutoff value of 3×10^3 . The concentrate was passed through an anti-UTI antibody-Sepharose column preliminarily equilibrated with 10 mM phosphate buffer (pH 7.5), whereby active fractions were adsorbed on the column. Then, after washing with 10 mM phosphate buffer (pH 7.5) containing 0.5 M NaCl, the active fractions were eluted with 0.1 M citric acid (pH 2.0) and 0.1 M citric acid containing 3 M potassium thiocyanate. The eluted active fractions were dialyzed against 10 mM phosphate buffer (pH 7.5) to obtain pure forms of the second region of human UTI having affinity for phosphatidylserine (rR-020C2) and the second region of unmodified human UTI (rR-020).

Example 16

Measuring Trypsin Inhibitory Activity

[0103] Each of the human UTI (rUTI), the second region of human UTI (rR-020), the human UTI having affinity for phosphatidylserine (rUTIC2) and the second region of human UTI having affinity for phosphatidylserine (rR-020C2), which are described in Examples 12 and 15, were expressed by COS-1 cells. To 100 µL of the supernatant of each culture medium, bovine trypsin (200 BAEEU/mL; product of Sigma) diluted with 0.2 M Tris-HCl buffer (pH 7.8) containing 0.1% BSA (said buffer is hereinafter designated as Buffer A) was added in an amount of 100 µL and reaction was performed at 25°C for 10 minutes; thereafter, 100 µL of a substrate solution containing 2 mg/mL of a synthetic substrate L-BAPA (product of Peptide Research Laboratory) dissolved in distilled water was added and reaction was performed at 25°C for 12 minutes. Subsequently, 100 µL of 50% aqueous acetic acid was added to terminate all reactions and the concentration of the synthetic substrate cleaved with the remaining trypsin was measured with a spectrophotometer (supra) at a wavelength of 405 nm. In the experiment, the second region of human UTI that was made up of the amino acid sequence of Sequence ID No. 23 and which had been expressed in *E. coli* and subsequently purified was used as a control. (see Japanese Patent Public Disclosure KOKAI 84083/1993)

Example 17

Measuring the Efficacy in Suppressing the Production of Active Oxygen

[0104] The amount of active oxygen produced from leukocytes was measured by a cytochrome C reduction method in accordance with the method of Kato et al. [Kato K. et al., Igaku To Yakugaku, Vol. 34, 499 (1995)]. Stated more specifically, leukocytes obtained from the peritoneal cavity of a rabbit were suspended in the physiological saline (pH 7.4) containing 2 mM glucose and 5 mM HEPES to give a concentration of 1×10^6 cells/mL; to 0.49 mL of the suspension, 0.5 mL of biological saline (pH 7.4) containing 132 µM cytochrome C, 2 mM glucose, 2 mM CaCl_2 , 5 mM HEPES and the supernatant of the culture of COS-1 cells having expressed therein the human UTI having affinity for phosphatidylserine (rUTIC2) or unmodified human UTI (rUTI), both being described in Example 12, was added and reacted at 37°C for 3 minutes. Then, 5 µL each of cytochalasin E (1 mg/mL) and concanavalin A (10 ng/mL) was added and the rate of increase in absorbance at a wavelength of 550 nm was measured with a spectrophotometer (supra).

[0105] The result is shown in Fig. 19, from which it was clear that the human UTI having affinity for phosphatidylserine (rUTIC2) according to the present invention was more effective than the unmodified human UTI (rUTI) in suppressing the production of active oxygen.

Example 18

Measuring the Effect on Prothrombinase Inhibition

[0106] Measurement of prothrombinase activity was measured by a procedure adapted from the method of Nesheim, M.E. [The Journal of Biological Chemistry, Vol. 254, 10952 (1979)]. Stated more specifically, 50 mM Tris-HCl buffer (pH 7.4) containing 6 µM factor Xa (product of American Diagnostica), 6 µM factor Va (product of Haematological Technol-

ogy), 0.15 M NaCl, 2 mM CaCl₂ and 0.1% BSA was provided (said buffer is hereinafter designated as Buffer B); to 100 μ L of Buffer B, 25 μ L of Buffer B containing liposomes by the content of 36 mg/mL in terms of phospholipid that were prepared by the same procedure as in Example 6 and which consisted of 25% phosphatidylserine and 75% phosphatidylcholine (except that TIBS was replaced by BSA-free Buffer B for the preparation of the liposomes) was added, and the mixture was heated at 37°C for 10 minutes. Then, the supernatant of the culture of COS-1 cells having expressed therein the second region of human UTI having affinity for phosphatidylserine (rR-020C2) as described in Example 15 or the second region of human UTI (supra) that was made up of the amino acid sequence of Sequence ID No. 23 and that was expressed in *E. coli* and subsequently purified was added optionally after dilution with the supernatant of the culture of COS-1 cells in an amount of 125 μ L, and the resulting mixture was heated at 37°C for 10 minutes. Then, 50 μ L of Buffer B containing 60 μ M of prothrombin (product of Enzyme Research Laboratory) was added and reaction was performed at 37°C for 30 minutes; thereafter, 300 μ L of buffer B containing 10 mM of EDTA was added to terminate the thrombin producing reaction. Subsequently, 100 μ L of the solution obtained by the aforementioned thrombin producing reaction which was no longer in progress was heated at 37°C for 10 minutes; thereafter, 100 μ L of 50 mM Tris-HCl buffer (pH 7.4) containing 1.5 mM synthetic substrate S-2238 (product of Daiichi Pure Chemicals Co., Ltd.), 0.15 M NaCl, 10 mM EDTA and 0.1% BSA was added and reaction was performed at 37°C for 10 minutes; thereafter, 100 μ L of 50% aqueous acetic acid was added to terminate all reactions and the concentration of the synthetic substrate cleaved with the produced thrombin was measured with a spectrophotometer (supra) at a wavelength of 405 nm. As it turned out, the second region of human UTI having affinity for phosphatidylserine (rR-020C2) according to the present invention was about 10 times more effective in inhibiting prothrombinase activity than the second region of human UTI made up of the amino acid sequence ID No. 23 (supra) that was expressed in *E. coli* and subsequently purified (see Fig. 20). It should be noted that there were no visible differences in activity between the second region of human UTI made up of the amino acid sequence of Sequence ID No. 23 that was expressed in *E. coli* and subsequently purified (supra) and the second region of the unmodified human UTI (rR-020) that was expressed in COS-1 cells.

Example 19

Specificity for A Specified Phospholipid, (2)

[0107] Liposomes were prepared by the same procedure as in Example 6 and using those liposomes, the second region of human UTI having affinity for phosphatidylserine (rR-020C2) and the second region of the unmodified human UTI (rR-020) were measured for their prothrombinase inhibitory activity in accordance with the method described in Example 18. The result is shown in Fig. 21, from which it was clear that the second region of human UTI having affinity for phosphatidylserine (rR-020C2) according to the present invention had its prothrombinase inhibitory activity accelerated in a manner specific for phosphatidylserine.

Example 20

Preparation of Plasmid Expressing Soluble Human MCP Having Affinity for Phosphatidylserine

[0108] Four species of single-stranded DNA (F17, F18, F19 and F20; see Fig. 5) were synthesized with a chemical synthesizer (supra) and F17 and F18 were annealed in the usual manner and so were F19 and F20, thereby yielding DNA fragment I which was ca. 60 bp in length and which had an Eco81I cleaved surface at 5' end and a HindIII cleaved surface at 3' end, as well as DNA fragment J which was ca. 90 bp in length and which had an NspV cleaved surface at 5' end and a KpnI cleaved surface at 3' end. Subsequently, the pUC19-sMCP (D) described in International Patent Publication WO/93/17122 was digested with restriction enzymes Eco81I and HindIII and subjected to agarose gel electrophoresis, thereby yielding a DNA fragment of ca. 3.6 kbp. This DNA fragment was ligated with fragment I in the usual manner to yield pUC19-sMCP(D)-C2-sub1. Subsequently, the pUC19-sMCP(D)-C2-sub1 was digested with restriction enzymes SphI and NspV and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 3.7 kbp. In a separate step, the pM1357 prepared in Example 3(2) was digested with restriction enzymes SphI and NspV and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 0.4 kbp. The two DNA fragments of ca. 3.7 kbp and Ca. 0.4 kbp in length were ligated in the usual manner to yield pUC19-sMCP(D)-C2-sub2. Subsequently, the pUC19-sMCP(D)-C2-sub2 was digested with restriction enzymes NspV and KpnI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.0 kbp. This DNA fragment was ligated with fragment J in the usual manner to yield pUC19-sMCP(D)-C2. Further, the pUC19-sMCP(D)-C2 was digested with restriction enzymes EcoRI and KpnI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of Ca. 1.5 kbp. In a separate step, the pM851 described in International Patent Publication WO/93/17122 was digested with restriction enzymes EcoRI and KpnI and subjected to agarose gel electrophoresis, thereby separating and recovering a DNA fragment of ca. 4.0 kbp. The two DNA frag-

ments ca. 1.5 kbp and ca. 4.0 kbp in length were ligated in the usual manner to yield pM1390 (the process of its construction is shown in Fig. 22). This plasmid contained DNA consisting of a nucleotide sequence in which the nucleotide sequence of Sequence ID No. 20 was linked to the 3' side of the nucleotide sequence of Sequence ID No. 29; using this plasmid, one can produce soluble human MCP having affinity for phosphatidylserine which is a peptide consisting of an amino acid sequence in which the amino acid sequence of Sequence ID No. 9 is linked to the C terminus of the amino acid sequence of Sequence ID No. 25 [said soluble human MCP is hereinafter designated as rsMCP(D)C2].

Example 21

Expression of rsMCP(D) and rsMCP(D)C2

[0109] The plasmid pM1390 prepared in Example 20 and the pM851 described in International Patent Publication WO/93/17122 were each transfected into COS-1 cells (supra) by a DEAE dextran method (supra), thereby expressing soluble human MCP having affinity for phosphatidylserine (rsMCP(D)C2) and unmodified soluble human MCP [rsMCP(D)]. Stated more specifically, Ca. 3×10^5 cells/9 cm² were inoculated in each plastic tissue culture plate and cultivated at 37°C for one day in 2 mL of a Dulbecco modified Eagle's medium (supra) containing 10% fetal bovine serum. After washing three times with 2 mL of DMEM, the culture medium was replaced by 0.7 mL of DMEM containing 1 µg of each of the plasmids, 50 mM Tris-HCl (pH 7.4), 0.2 mg/ml of DEAE dextran and 150 µM chloroquine. After cultivation at 37°C for 4 h, the culture solution was removed by suction; after washing once with 2 mL of DMEM and once with DMEM containing 10% fetal bovine serum, 2 mL of DMEM containing 10% fetal bovine serum was added and cultivation was continued at 37°C for 24 h. Thereafter, the culture medium was replaced by DMEM containing 0.1% BSA and cultivation was continued at 37°C for an additional 72 h, and the supernatant of the culture was collected. It was found that the collected culture medium contained soluble human MCP having affinity for phosphatidylserine [rsMCP(D)C2] and unmodified soluble human MCP [rsMCP(D)] in amounts of 1 - 5 µg/mL.

[0110] The collected culture solution was purified in accordance with the method described in International Patent Publication WO/93/17122 to yield in pure form of the soluble human MCP having affinity for phosphatidylserine [rsMCP(D)C2] and the unmodified soluble human MCP [rsMCP(D)].

Industrial Applicability

[0111] According to the present invention, there are provided a drug and a novel peptide that are useful as preventives and therapeutics of diseases involving coagulopathy, inflammations and immune response, as well as DNA necessary for producing them and a process for producing said drug.

SEQUENCE LISTING

5

Sequence ID No. : 1

10

Sequence Length : 130

Sequence Type : amino acid

15

Molecular Type : peptide (A1)

Sequence :

20

Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile

1

5

10

15

25

Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala

20

25

30

35

30

Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn Asn Pro

40

45

50

35

Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr

55

60

65

70

40

Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile

75

80

85

90

45

Ser Ser Ser Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val

95

100

105

50

Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp

110

115

120

125

55

Pro Pro Leu Leu

5 130

10 Sequence ID No. : 2

Sequence Length : 1 8

15 Sequence Type : amino acid

Molecular Type : peptide (A2)

20 Sequence :

Xaa Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

25 1 5 10 15

30 provided that Xaa is Thr or Leu.

35 Sequence ID No. : 3

Sequence Length : 1 2

40 Sequence Type : amino acid

Molecular Type : peptide (A3)

45 Sequence :

Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr

50 1 5 10

55

EP 0 965 597 A1

Sequence ID No. : 4

Sequence Length : 4 9 7

Sequence Type : amino acid

Molecular Type : peptide (rsTM)

Sequence :

Ala Pro Ala Glu Pro Gln Pro Gly Gly Ser Gln Cys Val Glu His Asp Cys Phe

1 5 10 15

Ala Leu Tyr Pro Gly Pro Ala Thr Phe Leu Asn Ala Ser Gln Ile Cys Asp Gly

20 25 30 35

Leu Arg Gly His Leu Met Thr Val Arg Ser Ser Val Ala Ala Asp Val Ile Ser

40 45 50

Leu Leu Leu Asn Gly Asp Gly Gly Val Gly Arg Arg Arg Leu Trp Ile Gly Leu

55 60 65 70

Gln Leu Pro Pro Gly Cys Gly Asp Pro Lys Arg Leu Gly Pro Leu Arg Gly Phe

75 80 85 90

Gln Trp Val Thr Gly Asp Asn Asn Thr Ser Tyr Ser Arg Trp Ala Arg Leu Asp

95 100 105

Leu Asn Gly Ala Pro Leu Cys Gly Pro Leu Cys Val Ala Val Ser Ala Ala Glu

110 115 120 125

Ala Thr Val Pro Ser Glu Pro Ile Trp Glu Glu Gln Gln Cys Glu Val Lys Ala

130 135 140

EP 0 965 597 A1

Asp Gly Phe Leu Cys Glu Phe His Phe Pro Ala Thr Cys Arg Pro Leu Ala Val

5 145 150 155 160

Glu Pro Gly Ala Ala Ala Ala Val Ser Ile Thr Tyr Gly Thr Pro Phe Ala

10 165 170 175 180

Ala Arg Gly Ala Asp Phe Gln Ala Leu Pro Val Gly Ser Ser Ala Ala Val Ala

15 185 190 195

Pro Leu Gly Leu Gln Leu Met Cys Thr Ala Pro Pro Gly Ala Val Gln Gly His

20 200 205 210 215

Trp Ala Arg Glu Ala Pro Gly Ala Trp Asp Cys Ser Val Glu Asn Gly Gly Cys

25 220 225 230

Glu His Ala Cys Asn Ala Ile Pro Gly Ala Pro Arg Cys Gln Cys Pro Ala Gly

30 235 240 245 250

Ala Ala Leu Gln Ala Asp Gly Arg Ser Cys Thr Ala Ser Ala Thr Gln Ser Cys

35 255 260 265 270

Asn Asp Leu Cys Glu His Phe Cys Val Pro Asn Pro Asp Gln Pro Gly Ser Tyr

40 275 280 285

Ser Cys Met Cys Glu Thr Gly Tyr Arg Leu Ala Ala Asp Gln His Arg Cys Glu

45 290 295 300 305

Asp Val Asp Asp Cys Ile Leu Glu Pro Ser Pro Cys Pro Gln Arg Cys Val Asn

50 310 315 320

55

EP 0 965 597 A1

Thr Gln Gly Gly Phe Glu Cys His Cys Tyr Pro Asn Tyr Asp Leu Val Asp Gly
 5 325 330 335 340
 Glu Cys Val Glu Pro Val Asp Pro Cys Phe Arg Ala Asn Cys Glu Tyr Gln Cys
 10 345 350 355 360
 Gln Pro Leu Asn Gln Thr Ser Tyr Leu Cys Val Cys Ala Glu Gly Phe Ala Pro
 15 365 370 375
 Ile Pro His Glu Pro His Arg Cys Gln Met Phe Cys Asn Gln Thr Ala Cys Pro
 20 380 385 390 395
 Ala Asp Cys Asp Pro Asn Thr Gln Ala Ser Cys Glu Cys Pro Glu Gly Tyr Ile
 25 400 405 410
 Leu Asp Asp Gly Phe Ile Cys Thr Asp Ile Asp Glu Cys Glu Asn Gly Gly Phe
 30 415 420 425 430
 Cys Ser Gly Val Cys His Asn Leu Pro Gly Thr Phe Glu Cys Ile Cys Gly Pro
 35 435 440 445 450
 Asp Ser Ala Leu Val Arg His Ile Gly Thr Asp Cys Asp Ser Gly Lys Val Asp
 40 455 460 465
 Gly Gly Asp Ser Gly Ser Gly Glu Pro Pro Pro Ser Pro Thr Pro Gly Ser Thr
 45 470 475 480 485
 Leu Thr Pro Pro Ala Val Gly Leu Val His Ser
 50 490 495
 55

EP 0 965 597 A1

Sequence ID No. : 5

Sequence Length : 30

Sequence Type : amino acid

Molecular Type : peptide (·)

Sequence :

Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

1 5 10 15

Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr

20 25 30

Sequence ID No. : 6

Sequence Length : 48

Sequence Type : amino acid

Molecular Type : peptide (Td)

Sequence :

Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

1 5 10 15

Leu Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

20 25 30 35

Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr

40 45

EP 0 965 597 A1

Sequence ID No. : 7

Sequence Length : 66

Sequence Type : amino acid

Molecular Type : peptide (Td-3)

Sequence :

Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

1 5 10 15

Leu Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

20 25 30 35

Leu Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

40 45 50

Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr

55 60 65

Sequence ID No. : 8

Sequence Length : 60

Sequence Type : amino acid

Molecular Type : peptide (C2D)

Sequence :

EP 0 965 597 A1

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe

5 1 5 10 15

Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile

10 20 25 30 35

His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys

15 40 45 50

Glu Ala Gln Asp Leu Tyr

20 55 60

25 Sequence ID No. : 9

Sequence Length : 1 6 0

30 Sequence Type : amino acid

Molecular Type : peptide (C2)

35 Sequence :

Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile

40 1 5 10 15

Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala

45 20 25 30 35

Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn Asn Pro

50 40 45 50

55

EP 0 965 597 A1

Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr

5 55 60 65 70

Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile

10 75 80 85 90

Ser Ser Ser Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val

15 95 100 105

Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp

20 110 115 120 125

Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln

25 130 135 140

Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr

30 145 150 155 160

35 Sequence ID No. : 1 0

Sequence Length : 3 0

40 Sequence Type : amino acid

Molecular Type : peptide (A1)

45 Sequence :

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe

50 1 5 10 15

55

EP 0 965 597 A1

Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu

5 20 25 30

10 Sequence ID No. : 1 1

Sequence Length : 3 9 0

15 Sequence Type : nucleic acid

Molecular Type : cDNA to mRNA (D1)

20 Sequence :

AGTTGCAGCA TGCCATTGGG AATGGAGAGT AAAGCAATAT CAGATGCACA GATTACTGCT 60

25 TCATCCTACT TTACCAATAT GTTGCCACC TGGTCTCCTT CAAAAGCTCG ACTTCACCTC 120

CAAGGGAGGA GTAATGCCTG GAGACCTCAG GTGAATAATC CAAAAGAGTG GCTGCAAGTG 180

30 GACTTCCAGA AGACAATGAA AGTCACAGGA GTAAC TACTC AGGGAGTAAA ATCTCTGCTT 240

ACCAGCATGT ATGTGAAGGA GTTCCTCATC TCCAGCAGTC AAGATGGCCA TCAGTGGACT 300

35 CTCTTTTTC AGAATGGCAA AGTAAAGGTT TTTCAGGGAA ATCAAGACTC CTTCACACCT 360

GTGGTGAACT CTCTAGACCC ACCGTTACTG 390

40 Sequence ID No. : 1 2

Sequence Length : 9 0

45 Sequence Type : nucleic acid

Molecular Type : cDNA to mRNA (D1)

50 Sequence :

55

EP 0 965 597 A1

CTCTTTTTC AGAATGGCAA AGTAAAGGTT TTTCAGGGAA ATCAAGACTC CTTACACCT 60

5 GTGGTGAACCT CTCTAGACCC ACCGTTACTG 90

10 Sequence ID No. : 1 3

Sequence Length : 5 4

15 Sequence Type : nucleic acid

Molecular Type : cDNA t o mRNA (D2)

20 Sequence :

ACTCGCTACC TTCGMATTCA CCCCCAGAGT TGGGTGCACC AGATTGCCCT GAGG 54

25 provided that M is A or C.

30 Sequence ID No. : 1 4

Sequence Length : 5 4

35 Sequence Type : nucleic acid

Molecular Type : cDNA t o mRNA (D2)

40 Sequence :

CTGCGCTACC TTCGMATTCA CCCCCAGAGT TGGGTGCACC AGATTGCCCT GAGG 54

45 provided that M is A or C.

50 Sequence ID No. : 1 5

Sequence Length : 3 6

55

EP 0 965 597 A1

Sequence Type : nucleic acid

5 Molecular Type : cDNA to mRNA (D3)

Sequence :

10 ATGGAGGTTC TGGGCTGCCA GGCACAGGAC CTCTAC

36

15 Sequence ID No. : 1 6

Sequence Length : 9 0

20 Sequence Type : nucleic acid

Molecular Type : cDNA to mRNA (·)

25 Sequence :

ACTCGCTACC TTCGAATTCA CCCCAGAGT TGGGTGCACC AGATTGCCCT GAGGATGGAG 60

30 GTTCTGGGCT GCGAGGCACA GGACCTCTAC

90

35 Sequence ID No. : 1 7

Sequence Length : 1 4 4

40 Sequence Type : nucleic acid

Molecular Type : cDNA to mRNA (Td)

45 Sequence :

ACTCGCTACC TTCGCATTCA CCCCAGAGT TGGGTGCACC AGATTGCCCT GAGGCTGCGC 60

TACCTTCGAA TTCACCCCA GAGTTGGGTG CACCAGATTG CCCTGAGGAT GGAGGTTCTG 120

50 GGCTGCGAGG CACAGGACCT CTAC

144

EP 0 965 597 A1

Sequence ID No. : 1 8

Sequence Length : 1 9 8

Sequence Type : nucleic acid

Molecular Type : cDNA t o mRNA (Td-3)

Sequence :

ACTCGCTACC TTCGCATTCA CCCCCAGAGT TGGGTGCACC AGATTGCCCT GAGGCTGCGC 60
TACCTTCGCA TTCACCCCCA GAGTTGGGTG CACCAGATTG CCCTGAGGCT GCGCTACCTT 120
CGAATTCACC CCCAGAGTTG GGTGCACCAG ATTGCCCTGA GGATGGAGGT TCTGGGCTGC 180
GAGGCACAGG ACCTCTAC 198

Sequence ID No. : 1 9

Sequence Length : 1 8 0

Sequence Type : nucleic acid

Molecular Type : cDNA t o mRNA (C2D)

Sequence :

CTCTTTTTTC AGAATGGCAA AGTAAAGGTT TTTCAGGGAA ATCAAGACTC CTTCACACCT 60
GTGGTGAACCT CTCTAGACCC ACCGTTACTG ACTCGCTACC TTCGAATTCA CCCCCAGAGT 120
TGGGTGCACC AGATTGCCCT GAGGATGGAG GTTCTGGGCT GCGAGGCACA GGACCTCTAC 180

Sequence ID No. : 2 0

Sequence Length : 4 8 0

EP 0 965 597 A1

Sequence Type : nucleic acid

5 Molecular Type : cDNA to mRNA (C2)

Sequence :

10 AGTTGCAGCA TGCCATTGGG AATGGAGAGT AAAGCAATAT CAGATGCACA GATTACTGCT 60
TCATCCTACT TTACCAATAT GTTTGCCACC TGGTCTCCTT CAAAAGCTCG ACTTCACCTC 120
15 CAAGGGAGGA GTAATGCCTG GAGACCTCAG GTGAATAATC CAAAAGAGTG GCTGCAAGTG 180
GACTTCCAGA AGACAATGAA AGTCACAGGA GTAAC TACTC AGGGAGTAAA ATCTCTGCTT 240
20 ACCAGCATGT ATGTGAAGGA GTTCCTCATC TCCAGCAGTC AAGATGGCCA TCAGTGGACT 300
CTCTTTTTC AGAATGGCAA AGTAAAGGTT TTTCAGGGAA ATCAAGACTC CTTACACCT 360
25 GTGGTGAAGT CTCTAGACCC ACCGTTACTG ACTCGCTACC TTCGAATTCA CCCCAGAGT 420
TGGGTGCACC AGATTGCCCT GAGGATGGAG GTTCTGGGCT GCGAGGCACA GGACCTCTAC 480

30 Sequence ID No. : 2 1

35 Sequence Length : 1 4 9 1

Sequence Type : nucleic acid

40 Molecular Type : cDNA to mRNA (rsTM)

Sequence :

45 GCTCCCGCAG AGCCGCAGCC GGGTGGCAGC CAGTGCCTCG AGCACGACTG CTTGCGCTC 60
TACCCGGGCC CCGCGACCTT CCTCAATGCC AGTCAGATCT GCGACGACT GCGGGGCCAC 120
50 CTAATGACAG TCGCTCCTC GGTGGCTGCC GATGTCATT CTTGCTACT GAACGGCGAC 180
GGCGGCGTTG GCCGCCGGCG CCTCTGGATC GGCCTGCAGC TGCCACCCGG CTGCGGCGAC 240

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CCCAAGCGCC TCGGGCCCCT GCGCGGCTTC CAGTGGGTTA CGGGAGACAA CAACACCAGC 300

5 TATAGCAGGT GGGCACGGCT CGACCTCAAT GGGGCTCCCC TCTGCGGCC GTTGTGCGTC 360

GCTGTCTCCG CTGCTGAGG CACTGTGCCC AGCGAGCCGA TCTGGGAGGA GCAGCAGTGC 420

10 GAAGTGAAGG CCGATGGCTT CCTCTGCGAG TTCCACTTCC CAGCCACCTG CAGGCCACTG 480

GCTGTGGAGC CCGGCGCCGC GGCTGCCGCC GTCTCGATCA CCTACGGCAC CCCGTTGCGC 540

15 GCGCGCGGAG CGGACTTCCA GCGGCTGCCG GTGGGCAGCT CCGCCGCGGT GGCTCCCCTC 600

GGCTTACAGC TAATGTGCAC CGCGCCGCC GGAGCGGTCC AGGGGCACTG GGCCAGGGAG 660

20 GCGCCGGGCG CTTGGGACTG CAGCGTGGAG AACGGCGGCT GCGAGCACGC GTGCAATGCG 720

ATCCCTGGGG CTCCCCGCTG CCAGTGCCCA GCCGGCGCCG CCCTGCAGGC AGACGGGCGC 780

25 TCCTGCACCG CATCCGCGAC GCAGTCCTGC AACGACCTCT GCGAGCACTT CTGCGTTCCC 840

AACCCCGACC AGCCGGGCTC CTACTCGTGC ATGTGCGAGA CCGGCTACCG GCTGGCGGCC 900

30 GACCAACACC GGTGCGAGGA CGTGGATGAC TGCATACTGG AGCCCAGTCC GTGTCCGCAG 960

CGCTGTGTCA ACACACAGGG TGGCTTCGAG TGCCACTGCT ACCCTAACTA CGACCTGGTG 1020

35 GACGGCGAGT GTGTCGAGCC CGTGGACCCG TGCTTCAGAG CCAACTGCGA GTACCAGTGC 1080

CAGCCCCTGA ACCAACTAG CTACCTCTGC GTCTGCGCCG AGGGCTTCGC GCCCATTCCC 1140

40 CACGAGCCGC ACAGGTGCCA GATGTTTTGC AACCAGACTG CCTGTCCAGC CGACTGCGAC 1200

CCCAACACCC AGGCTAGCTG TGAGTGCCCT GAAGGCTACA TCCTGGACGA CGGTTTCATC 1260

45 TGCACGGACA TCGACGAGTG CGAAAACGGC GGCTTCTGCT CCGGGGTGTG CCACAACCTC 1320

CCCGGTACCT TCGAGTGCAT CTGCGGGCCC GACTCGGCC TTGTCCGCCA CATTGGCACC 1380

50 GACTGTGACT CCGGCAAGGT GGACGGTGGC GACAGCGGCT CTGGCGAGCC CCCGCCAGC 1440

CCGACGCCCC GCTCCACCTT GACTCCTCCG GCCGTGGGGC TCGTGCATTC G 1491

55

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Sequence ID No. : 2 2

Sequence Length : 3 6

Sequence Type : amino acid

Molecular Type : peptide (TdD)

Sequence :

Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

1 5 10 15

Leu Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg

20 25 30 35

Sequence ID No. : 2 3

Sequence Length : 6 8

Sequence Type : amino acid

Molecular Type : peptide (second region of UTI)

Sequence :

Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu

1 5 10 15

Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly Cys

20 25 30 35

Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg Glu Tyr Cys Gly

40 45 50

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Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg Phe Ser Asn

5 55 60 65

10 Sequence ID No. : 2 4

Sequence Length : 1 4 7

15 Sequence Type : amino acid

Molecular Type : peptide (rUTI)

20 Sequence :

Ala Val Leu Pro Gln Glu Glu Gly Ser Gly Gly Gly Gln Leu Val Thr Glu

25 1 5 10 15

Val Thr Lys Lys Glu Asp Ser Cys Gln Leu Gly Tyr Ser Ala Gly Pro Cys Met

30 20 25 30 35

Gly Met Thr Ser Arg Tyr Phe Tyr Asn Gly Thr Ser Met Ala Cys Glu Thr Phe

35 40 45 50

Gln Tyr Gly Gly Cys Met Gly Asn Gly Asn Asn Phe Val Thr Glu Lys Glu Cys

40 55 60 65 70

Leu Gln Thr Cys Arg Thr Val Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro

45 75 80 85 90

Cys Arg Ala Phe Ile Gln Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val

50 95 100 105

55

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Leu Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys

5 110 115 120 125

Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg

10 130 135 140

Phe Ser Asn

15 145

20 Sequence ID No. : 2 5

Sequence Length : 2 7 9

25 Sequence Type : amino acid

Molecular Type : peptide (rsMCP)

30 Sequence :

Cys Glu Glu Pro Pro Thr Phe Glu Ala Met Glu Leu Ile Gly Lys Pro Lys Pro

35 1 5 10 15

Tyr Tyr Glu Ile Gly Glu Arg Val Asp Tyr Lys Cys Lys Lys Gly Tyr Phe Tyr

40 20 25 30 35

Ile Pro Pro Leu Ala Thr His Thr Ile Cys Asp Arg Asn His Thr Trp Leu Pro

45 40 45 50

Val Ser Asp Asp Ala Cys Tyr Arg Glu Thr Cys Pro Tyr Ile Arg Asp Pro Leu

50 55 60 65 70

55

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	Asn Gly Gln Ala Val Pro Ala Asn Gly Thr Tyr Glu Phe Gly Tyr Gln Met His
5	75 80 85 90
	Phe Ile Cys Asn Glu Gly Tyr Tyr Leu Ile Gly Glu Glu Ile Leu Tyr Cys Glu
10	95 100 105
	Leu Lys Gly Ser Val Ala Ile Trp Ser Gly Lys Pro Pro Ile Cys Glu Lys Val
15	110 115 120 125
	Leu Cys Thr Pro Pro Pro Lys Ile Lys Asn Gly Lys His Thr Phe Ser Glu Val
20	130 135 140
	Glu Val Phe Glu Tyr Leu Asp Ala Val Thr Tyr Ser Cys Asp Pro Ala Pro Gly
25	145 150 155 160
	Pro Asp Pro Phe Ser Leu Ile Gly Glu Ser Thr Ile Tyr Cys Gly Asp Asn Ser
30	165 170 175 180
	Val Trp Ser Arg Ala Ala Pro Glu Cys Lys Val Val Lys Cys Arg Phe Pro Val
35	185 190 195
	Val Glu Asn Gly Lys Gln Ile Ser Gly Phe Gly Lys Lys Phe Tyr Tyr Lys Ala
40	200 205 210 215
	Thr Val Met Phe Glu Cys Asp Lys Gly Phe Tyr Leu Asp Gly Ser Asp Thr Ile
45	220 225 230
	Val Cys Asp Ser Asn Ser Thr Trp Asp Pro Pro Val Pro Lys Cys Leu Lys Gly
50	235 240 245 250
55	

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Pro Arg Pro Thr Tyr Lys Pro Pro Val Ser Asn Tyr Pro Gly Tyr Pro Lys Pro

5 255 260 265 270

Glu Glu Gly Ile Leu Asp Ser Leu Asp

10 275

15 Sequence ID No. : 2 6

Sequence Length : 1 0 8

20 Sequence Type : nucleic acid

Molecular Type : cDNA to mRNA (TdD)

25 ACTCGCTACC TTCGCATTCA CCCCAGAGT TGGGTGCACC AGATTGCCCT GAGGCTGCGC 60

TACCTTCGAA TTCACCCCCA GAGTTGGGTG CACCAGATTG CCCTGAGG 108

30

Sequence ID No. : 2 7

35 Sequence Length : 2 0 4

Sequence Type : nucleic acid

40 Molecular Type : cDNA to mRNA (second region of UTI)

GCGGCCTGCA ATCTCCCCAT AGTCCGGGGC CCCTGCCGAG CCTTCATCCA GCTCTGGGCA 60

45 TTTGATGCTG TCAAGGGGAA GTGCGTCCTC TTCCCCTACG GGGGCTGCCA GGGCAACGGG 120

AACAAGTTCT ACTCAGAGAA GGAGTGCAGA GAGTACTGCG GTGTCCCTGG TGATGGTGAT 180

50 GAGGAGCTGC TGCGCTTCTC CAAC 204

55

EP 0 965 597 A1

Sequence ID No. : 2 8

Sequence Length : 4 4 1

Sequence Type : nucleic acid

Molecular Type : cDNA to mRNA (rUTI)

GCTGTGCTAC CCCAAGAAGA GGAAGGATCA GGGGGTGGGC AACTGGTAAC TGAAGTCACC 60
AAGAAAGAAG ATTCCTGCCA GCTGGGCTAC TCGGCCGGTC CCTGCATGGG AATGACCAGC 120
AGGTATTTCT ATAATGGTAC ATCCATGGCC TGTGAGACTT TCCAGTACGG CGGCTGCATG 180
GGCAACGGTA ACAACTTCGT CACAGAAAAG GAGTGTCTGC AGACCTGCCG AACTGTGGCG 240
GCCTGCAATC TCCCCATAGT CCGGGGCCCC TGCCGAGCCT TCATCCAGCT CTGGGCATTT 300
GATGCTGTCA AGGGGAAGTG CGTCCTCTTC CCCTACGGGG GCTGCCAGGG CAACGGGAAC 360
AAGTTCTACT CAGAGAAGGA GTGCAGAGAG TACTGCGGTG TCCCTGGTGA TGGTGATGAG 420
GAGCTGCTGC GCTTCTCCAA C 441

Sequence ID No. : 2 9

Sequence Length : 8 3 7

Sequence Type : nucleic acid

Molecular Type : cDNA to mRNA (rsMCP)

TGTGAGGA GCCACCAACA TTTGAAGCTA TGGAGCTCAT TGGTAAACCA AAACCCTACT 58
ATGAGATTGG TGAACGAGTA GATTATAAGT GTAAAAAAGG ATACTTCTAT ATACCTCCTC 118
TTGCCACCCA TACTATTTGT GATCGGAATC ATACATGGCT ACCTGTCTCA GATGACGCCT 178
GTTATAGAGA AACATGTCCA TATATACGGG ATCCTTTAAA TGGCCAAGCA GTCCCTGCAA 238

ATGGGACTTA CGAGTTTGGT TATCAGATGC ACTTTATTTG TAATGAGGGT TATTACTTAA 298

5 TTGGTGAAGA AATTCTATAT TGTGAACTTA AAGGATCAGT AGCAATTTGG AGCGGTAAGC 358

CCCCAATATG TGAAAAGGTT TTGTGTACAC CACCTCCAAA AATAAAAAAT GGAAAAACACA 418

10 CCTTTAGTGA AGTAGAAGTA TTTGAGTATC TTGATGCAGT AACTTATAGT TGTGATCCTG 478

CACCTGGACC AGATCCATTT TCACTTATTG GAGAGAGCAC GATTTATTGT GGTGACAATT 538

15 CAGTGTGGAG TCGTGCTGCT CCAGAGTGTA AAGTGGTCAA ATGTCGATTT CCAGTAGTCG 598

AAAATGGAAA ACAGATATCA GGATTGGA AAAAATTTTA CTACAAAGCA ACAGTTATGT 658

20 TTGAATGCGA TAAGGGTTTT TACCTCGATG GCAGCGACAC AATTGTCTGT GACAGTAACA 718

GTACTTGGGA TCCCCAGTT CCAAAGTGTC TTAAAGGTCC TAGGCCTACT TACAAGCCTC 778

25 CAGTCTCAAA TTATCCAGGA TATCCTAAAC CTGAGGAAGG AATACTTGAC AGTTTGGAT 837

Claims

1. A drug containing both a substance having affinity for a phospholipid and a biologically active substance.
2. The drug according to claim 1, wherein said phospholipid is one that is contained in the constituents of lipid bilayers forming the surface layers of cells.
3. The drug according to claim 1 or 2, wherein said phospholipid is one of which the proportion in the outer part of each of the lipid bilayers forming the surface layers of cells increases when the cell is not normal.
4. The drug according to any one of claims 1 - 3, wherein said phospholipid is phosphatidylserine or phosphatidylethanolamine.
5. The drug according to any one of claims 1 - 4, wherein said phospholipid is phosphatidylserine.
6. The drug according to any one of claims 1 - 5, wherein said substance having affinity for a phospholipid has a sequence represented by the following general formula:

$$(A1)n_1 - (A2)n_2 - (A3)n_3$$
 (provided that A1 is the amino acid sequence denoted by Sequence ID No. 1 or 10; A2 and A3 are the amino acid sequences denoted by Sequence ID Nos. 2 and 3, respectively; n_1 , n_2 and n_3 which are the repeating numbers of A1, A2 and A3, respectively, are 0 - 5, 1 - 5 and 0 - 5, respectively.)
7. The drug according to claim 6, wherein said n_1 is 0 or 1, n_2 is 1, 2 or 3, and n_3 is 0 or 1.
8. The drug according to claim 6 or 7, wherein said substance having affinity for a phospholipid has either one of the following sequences:

(A1)₁ - (A2)₁ - (A3)₁

5 (A2)₁ - (A3)₁

(A2)₂ - (A3)₁

10 (A2)₃ - (A3)₁

(A2)₂

15

9. The drug according to any one of claims 6 - 8, wherein said substance having affinity for a phospholipid has either one of the following sequences:

20

(A2)₂ - (A3)₁

(A2)₃ - (A3)₁

25

(A2)₂

30

10. The drug according to any one of claims 1 - 9, wherein said biologically active substance is a peptide.

11. The drug according to claim 10, wherein said peptide is at least one member of the group consisting of a factor involved in a blood coagulation system, a factor involved in a fibrinolytic system, a factor involved in an immune response reaction, a factor suppressing cytopathy, and a factor inhibiting the activity of proteases.

35

12. The drug according to claim 10, wherein said peptide is at least one member of the group consisting of a factor suppressing a blood coagulation, a factor enhancing a fibrinolytic system, a factor suppressing a complement activating reaction, a factor suppressing the cytopathy due to active oxygen, and a factor inhibiting the activity of proteases.

40

13. The drug according to any one of claims 10 - 12, wherein said peptide is thrombomodulin (TM), the second region of ulinastatin (UTI), a membrane cofactor protein (MCP), UTI or a modified version thereof.

45

14. The drug according to any one of claims 10 - 13, wherein said peptide has the amino acid sequence denoted by Sequence ID No. 4 or either of Sequence ID Nos. 23 - 25.

15. The drug according to any one of claims 10 - 14, wherein said biologically active substance and said substance having affinity for a phospholipid are bound via a peptide bond.

50

16. The drug according to any one of claims 10 - 15, wherein the N terminal amino acid of said substance having affinity for a phospholipid and the C terminal amino acid of said biologically active substance are bound via a peptide bond.

55

17. A peptide having the amino acid sequence represented by the following general formula:

(A2)_{n₂} - (A3)_{n₃}

(provided that A2 and A3 are the amino acid sequences denoted by Sequence ID Nos. 2 and 3, respectively; n₂ and n₃ which represent the repeating number of A2 and A3, respectively, are 2 or 3 and 0 or 1, respectively.)

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18. The peptide according to claim 17, wherein said amino acid sequence has either one of the following sequences:

$(A2)_2 - (A3)_1$

$(A2)_3 - (A3)_1$

$(A2)_2$

19. DNA coding for the amino acid sequence of the drug according to claim 15 or 16.

20. DNA coding for the amino acid sequence of the peptide according to claim 17 or 18.

21. A process for producing the drug according to any one of claims 1 - 16.

FIG. 1

S 1 : TTGTCGACAT GCTTGGGGTC CTGGTCCTT

S 2 : ATAAGCTTCC GCTGCTGAGG CCACTGTGC

S 3 : TTCTGCAGCT CGAGCCCGTG GACCCGTGC TTC

S 4 : GCATTGCGAG CACTCTTCGC ATGG

S 5 : AAAATGCATT CGAGTTGCAG CATGCCATTG GCAATGG

S 6 : AAAATGCATT CGCTCTTTTT TCAGAATGGC AAAGTAAAGG

S 7 : TTCAAGCTTA TGAGGAGCCT CGGGGCCCTG CTCTTGCTG

S 8 : GCTCTAGAAT GAGGAGCCTC GGGGCCCTGC TCTTGCTGCT GAGCGCCTGC CTGGCCGTGA
GCGCTGCTGT GCTACCCCAA GAAGAGGAAG

S 9 : AAAGTGCAGA GAGTACTCGG GTGTCCCTGC TGATCGTGAT GAGGAGCTGC TCGCCTTCTC
CAACAGTTGC ACCATGCCAT TGGGAATCG

FIG. 2

A 1 : TTGGATCCCA CAGTGGCCTC AGCAGCGGA

A 2 : ATGTCGACAC ACTCGCCGTC CACCAGGTC

A 3 : AGAATTCCGA TCCTCAGAGT CTCTGCGGCG TCCGCTC

A 4 : GATAGTTAAT TCAGGAGGCT TC

A 5 : AAAAGAATTC TCGAGTCAGT AGAGGTCCTG TGCCTCGCAG CCCAGAACC

A 6 : CGCGGATCCT CAGTTGGAGA AGCGCAGCAG CTCCTCATC

A 7 : ATAAGAATGC GCGCGCTCAG TTGGAGAAGC GCAGCAGCTC CTC

A 8 : TTTGCGGCCC CTCAGTAGAG GTCCTGTGCC TCGC

FIG. 3

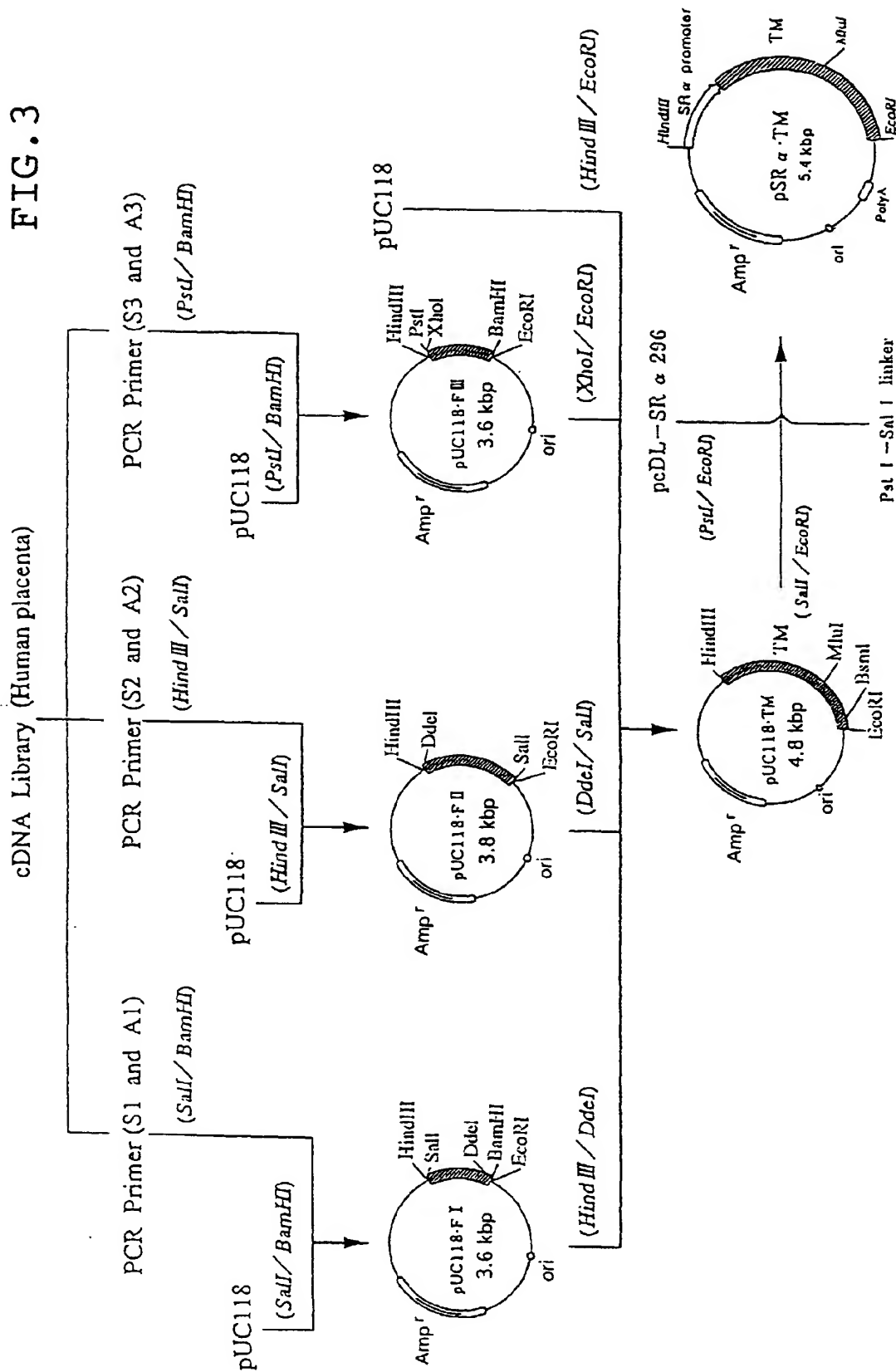


FIG. 4

F 1 : CATTGACTC GCTACCTTCG AACTCGAG

 F 2 : AATTCTCGAG TTCGAAGGTA GCGAGTCGAA TGCA

 F 3 : CGAATTCACC CCCAGAGTTG GGTGCACCAG ATTGCCCTGA GGATGGAGGT TCTGGGCTGC
 GAGGCACAGG ACCTCTACTG AGGTACCG

 F 4 : AATTGGGTAC CTCAGTAGAG GTCCTGTGCC TGCAGCCCA GAACCTCCAT CCTCAGGGCA
 ATCTGGTGCA CCCAACTCTG GGGGTGAATT

 F 5 : CGCATTACACC CCCAGAGTTG GGTGCACCAG ATTGCCCTGA GGCTGGGCTA CCTTCGAAGC
 TTG

 F 6 : AATTCAAGCT TCGAAGGTAG CGCAGCCTCA GGGCAATCTG GTGCACCCAA CTCTGGGGGT
 GAATG

 F 7 : CGAATTCACC CCCAGAGTTG GGTGCACCAG ATTGCCCTGA GGATGGAGGT TCTGGGCTGC
 GAGGCACAGG ACCTCTACTG AGGTACCG

 F 8 : AATTGGGTAC CTCAGTAGAG GTCCTGTGCC TGCAGCCCA GAACCTCCAT CCTCAGGGCA
 ATCTGGTGCA CCCAACTCTG GGGGTGAATT

 F 9 : CGAATTCACC CCCAGAGTTG GGTGCACCAG ATTGCCCTGA GGTGAGGTAC CG

 F 1 0 : AATTGGGTAC CTCACCTCAG GGCAATCTGG TGCACCCAAC TCTGGGGGTG AATT

FIG. 5

F 1 1 : CATTGCTGAG

F 1 2 : AATTCTCAGC AATGCA

F 1 3 : AATTCAGCGC TGTGCAGAGA GACTGCGGT GTCCCTGCAT GCGCATTGAG GACCTGGTAC
CGCGCCCGCA

F 1 4 : AGCTTGCGGC CGCGGTACCA GGTCTGAAT GCGCATGCAG GGACACCGCA GTACTCTCTG
CACACCGCTG

F 1 5 : GCTGCTGTGC TACCCAAGA AGAGGAAGGA GATGGGGCGG CCTGCAATCT CCCCATAGTC
CGGGGCC

F 1 6 : CCGCACTATG CGGAGATTGC AGGCGGCCCC ATCTCCTTCC TCTTCTTGGG GTAGCACAGC
AGC

F 1 7 : TGAGGAAGGA ATACTTGACA GTTTGAGTTG CAGCATGCAA AATTGAAAA AAGGTACCA

F 1 8 : AGCTTGCTAC CTTTTTTCGA ATTTTGCATG CTGCAACTCA AACTGTCAAG TATTCCTTCC

F 1 9 : CGAATCCACC CCCAGACTTG CGTGCACCAG ATTGCCCTGA CGATGGAGCT TCTGGGCTGC
GAGGCACAGG ACCTCTACTG AAGCTTGGA C

F 2 0 : CAAGCTTCAG TAGAGTCTCT GTGCCTCGCA GCCCAGAACC TCCATCCTCA GGGCAATCTG
GTGCACCCAA CTCTCGGGGT GCATT

FIG. 6

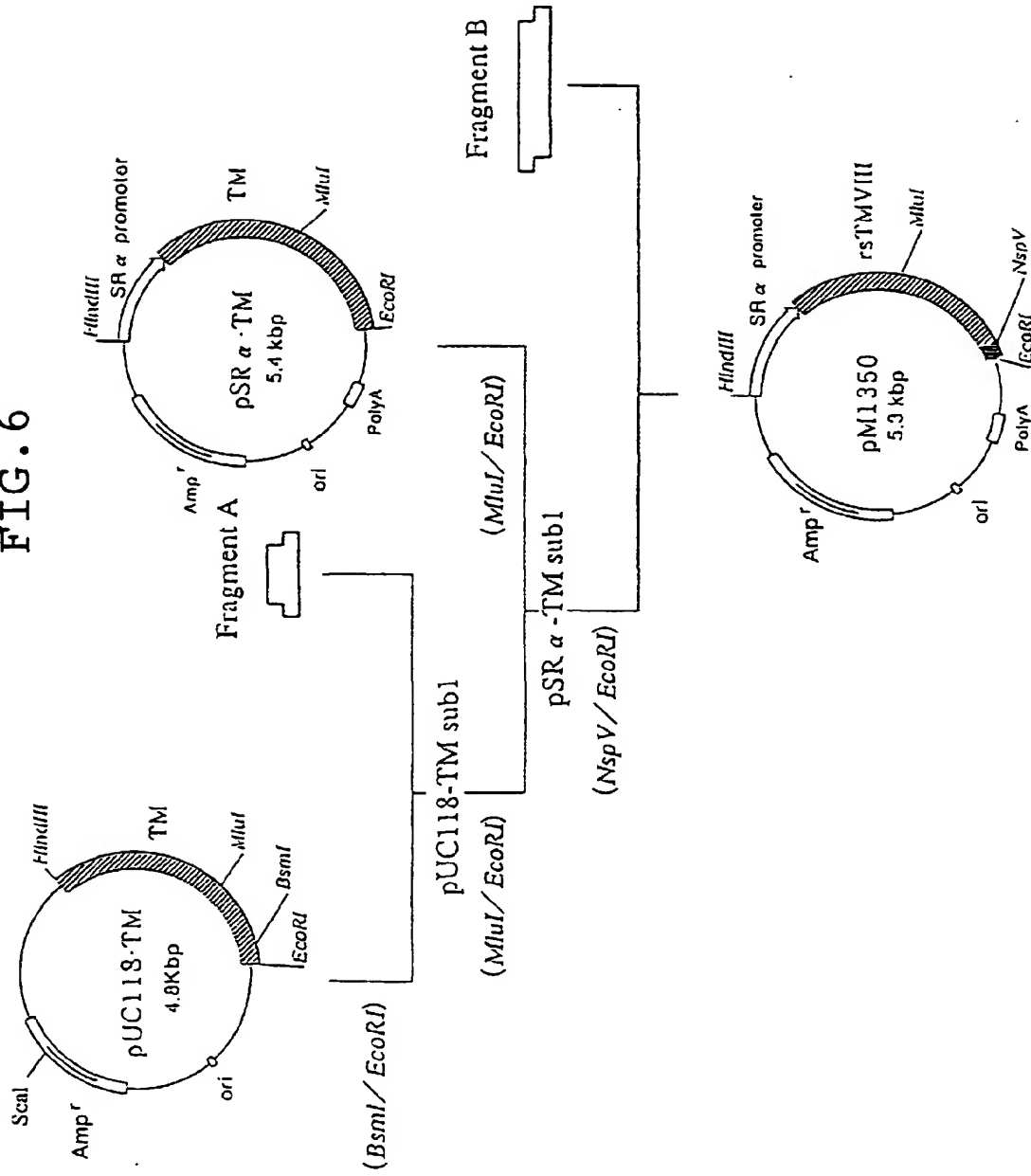


FIG. 7

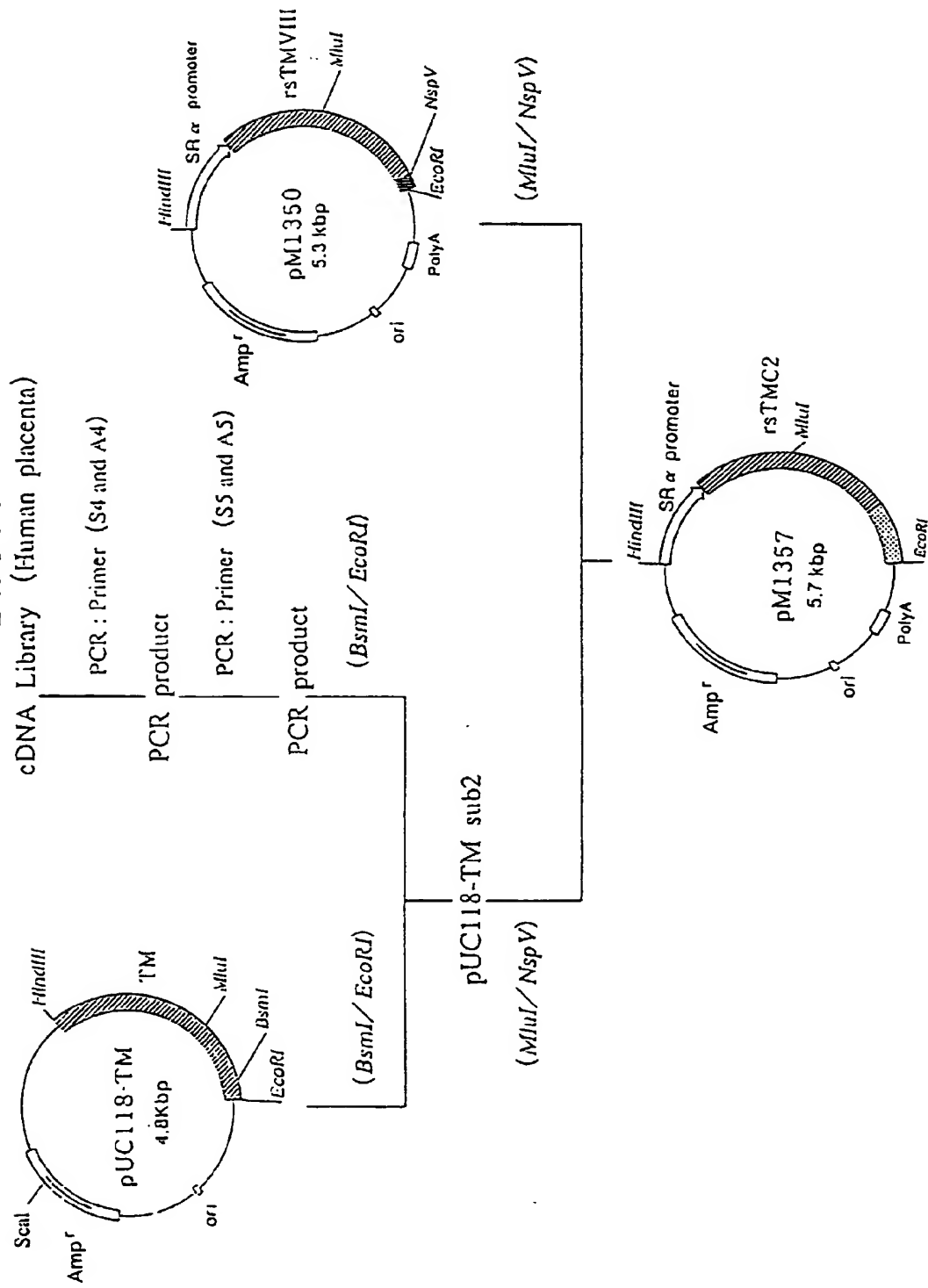


FIG. 8

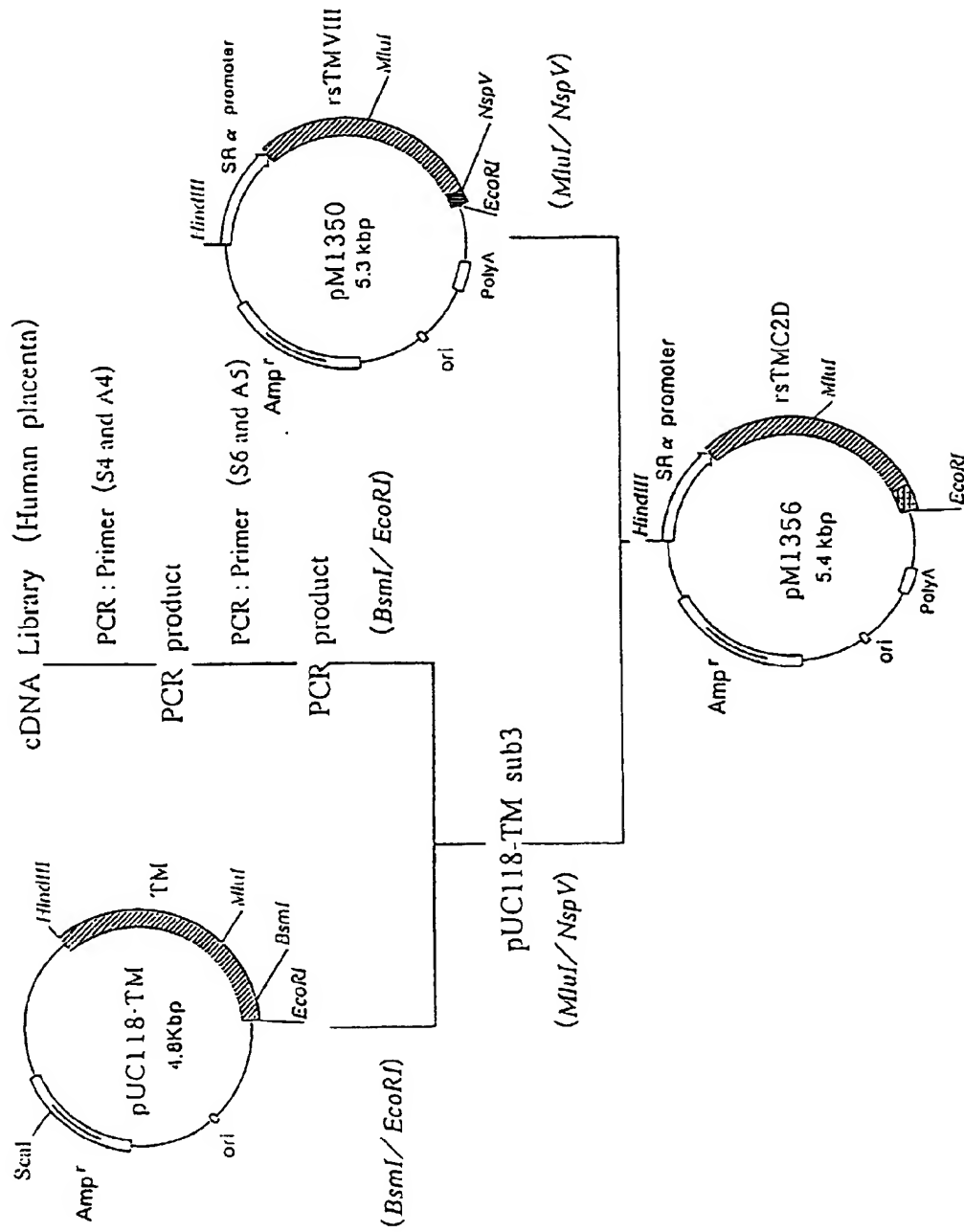


FIG. 9

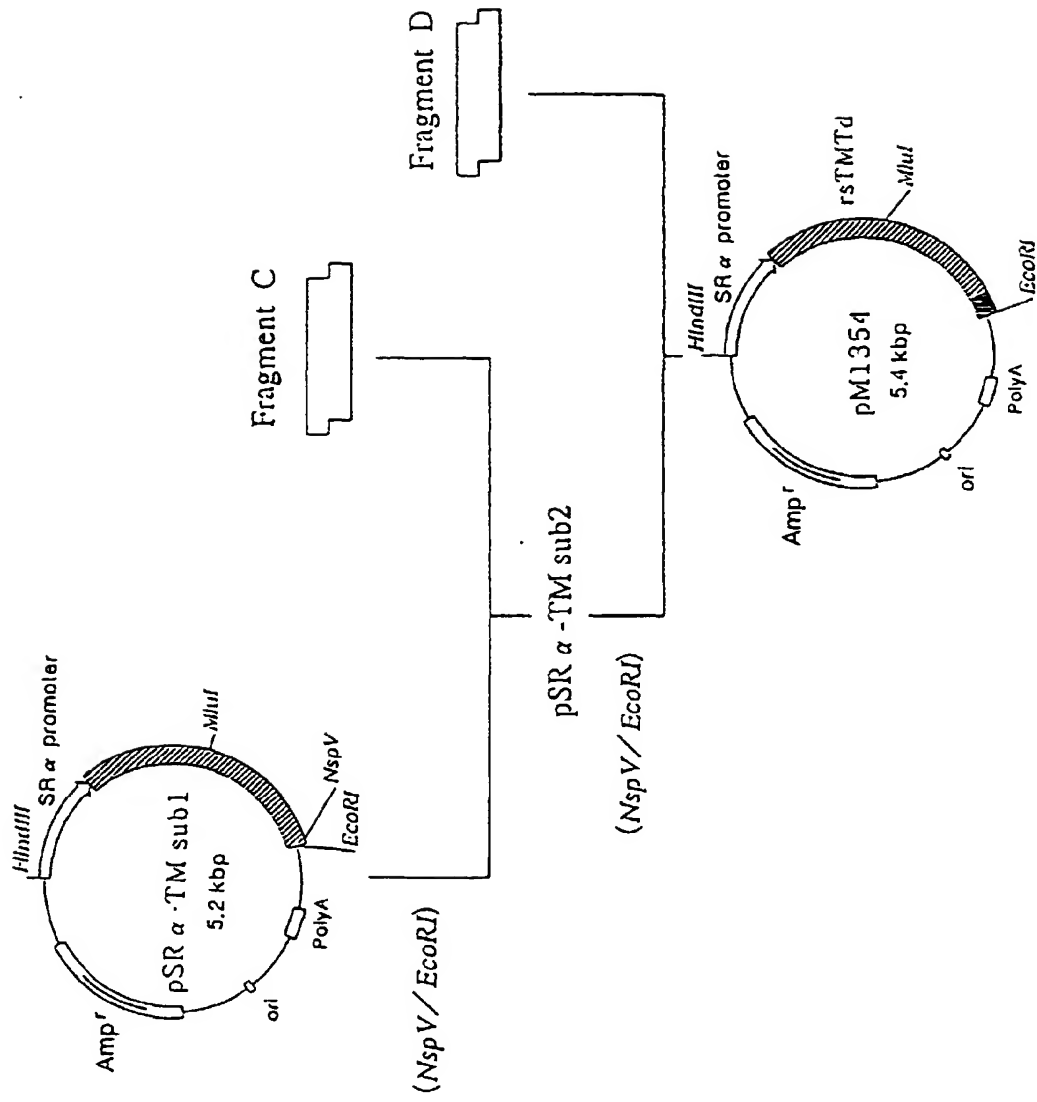


FIG. 10

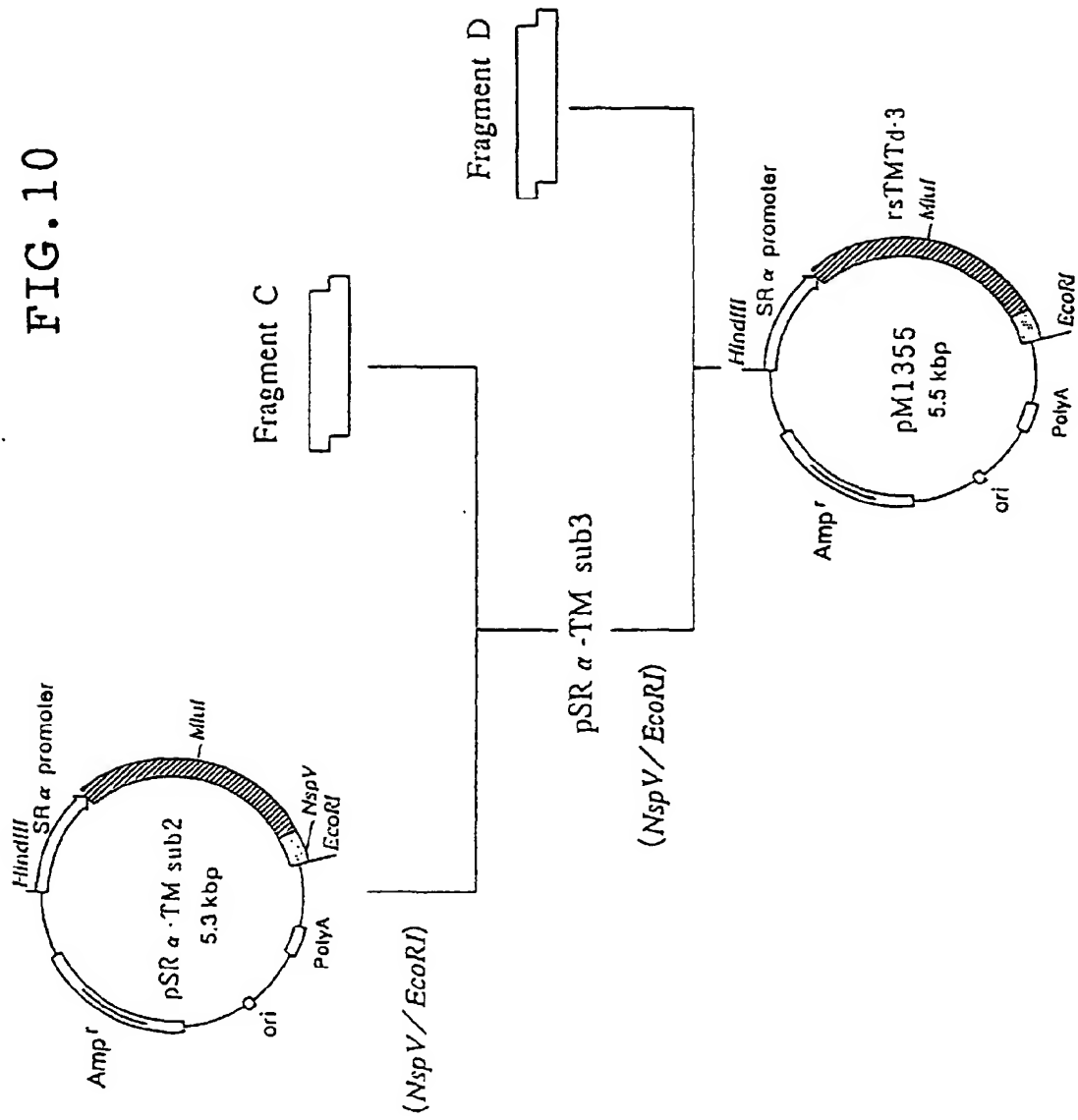


FIG. 11

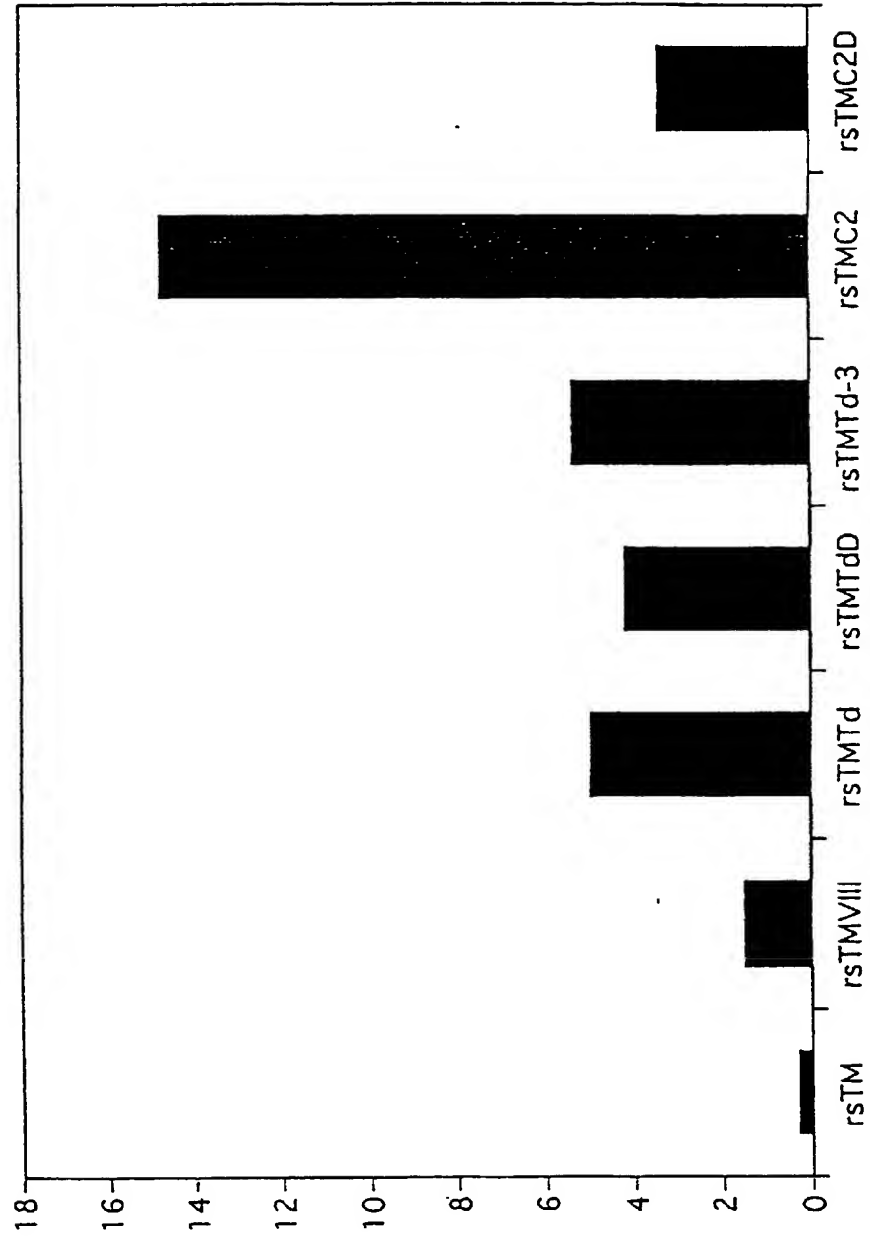


FIG.12

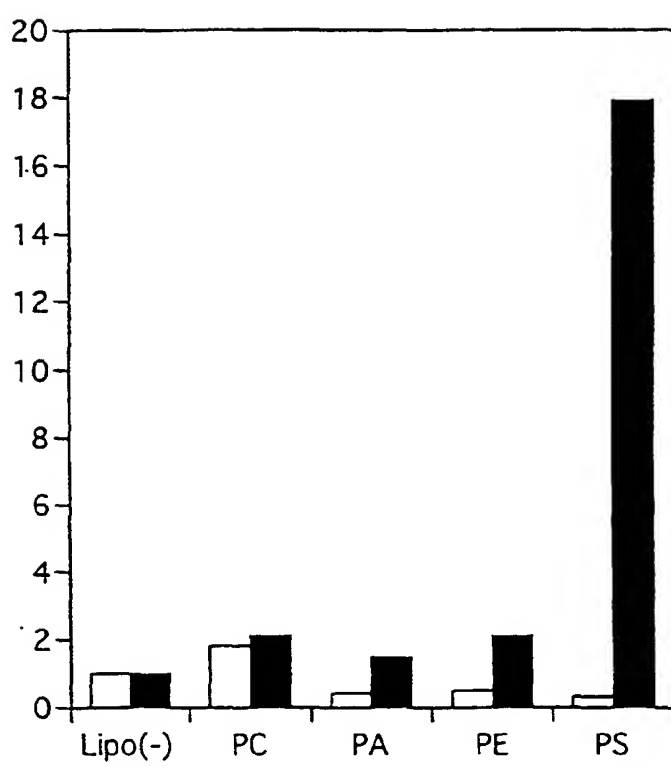


FIG. 13

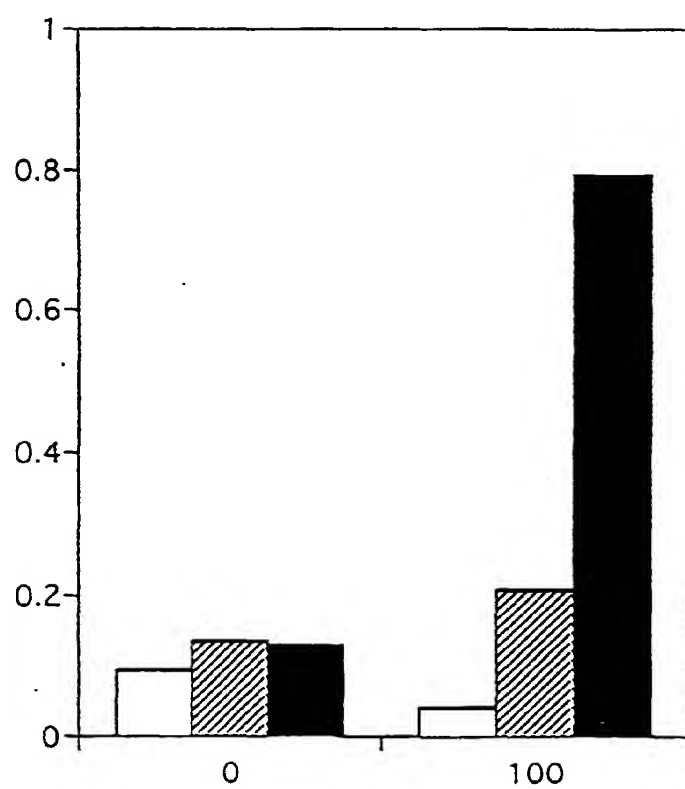
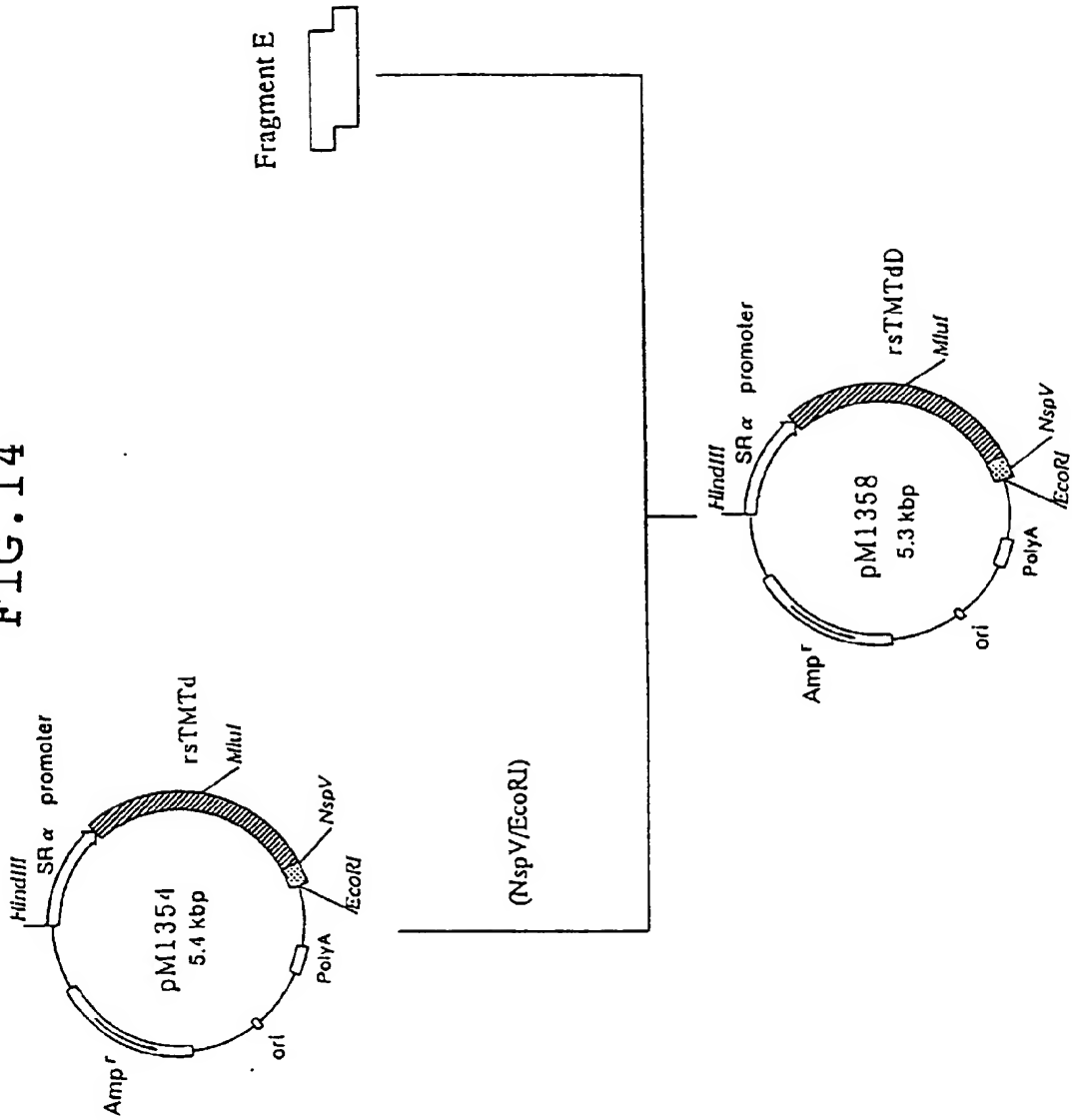
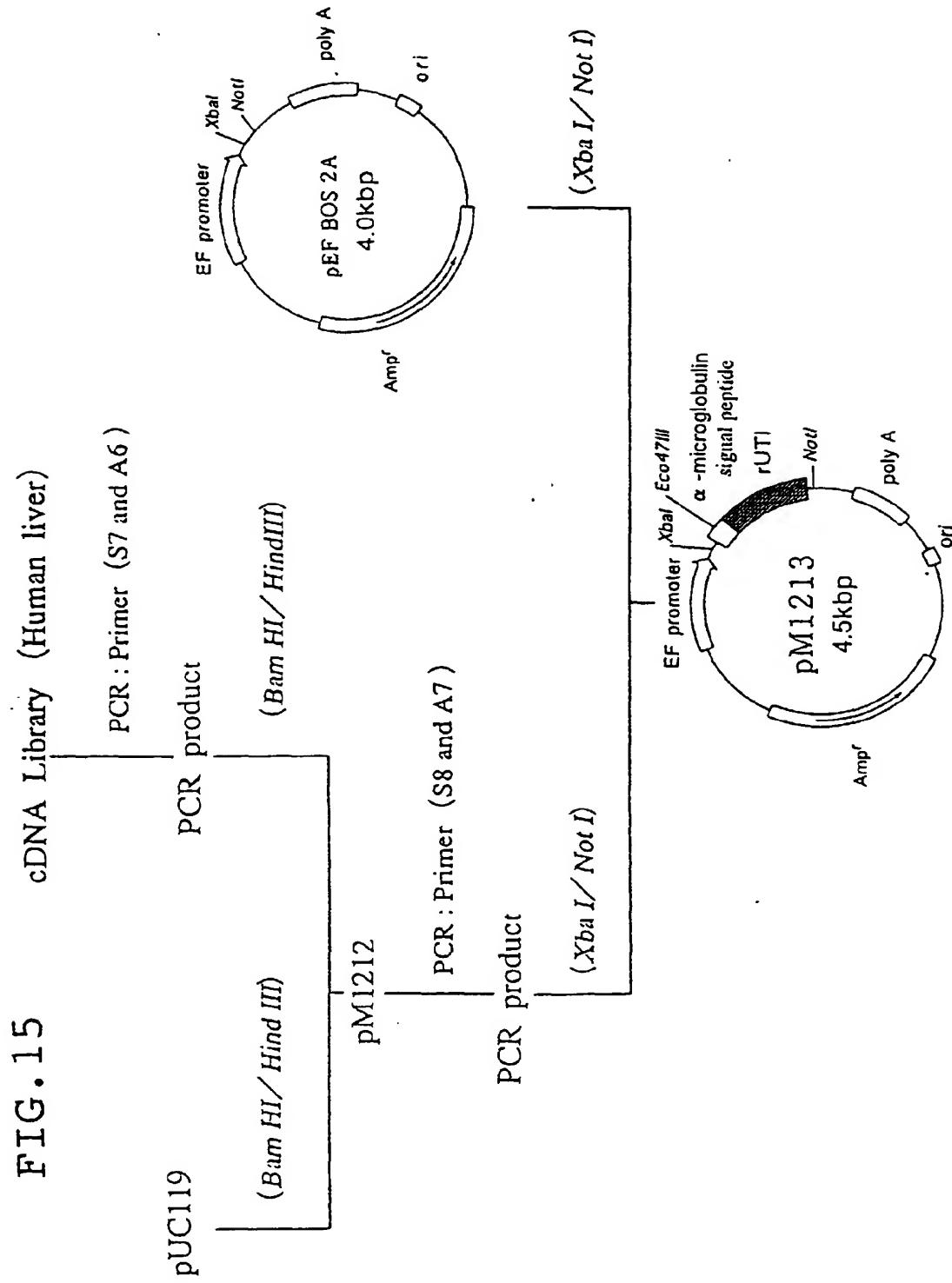


FIG. 14





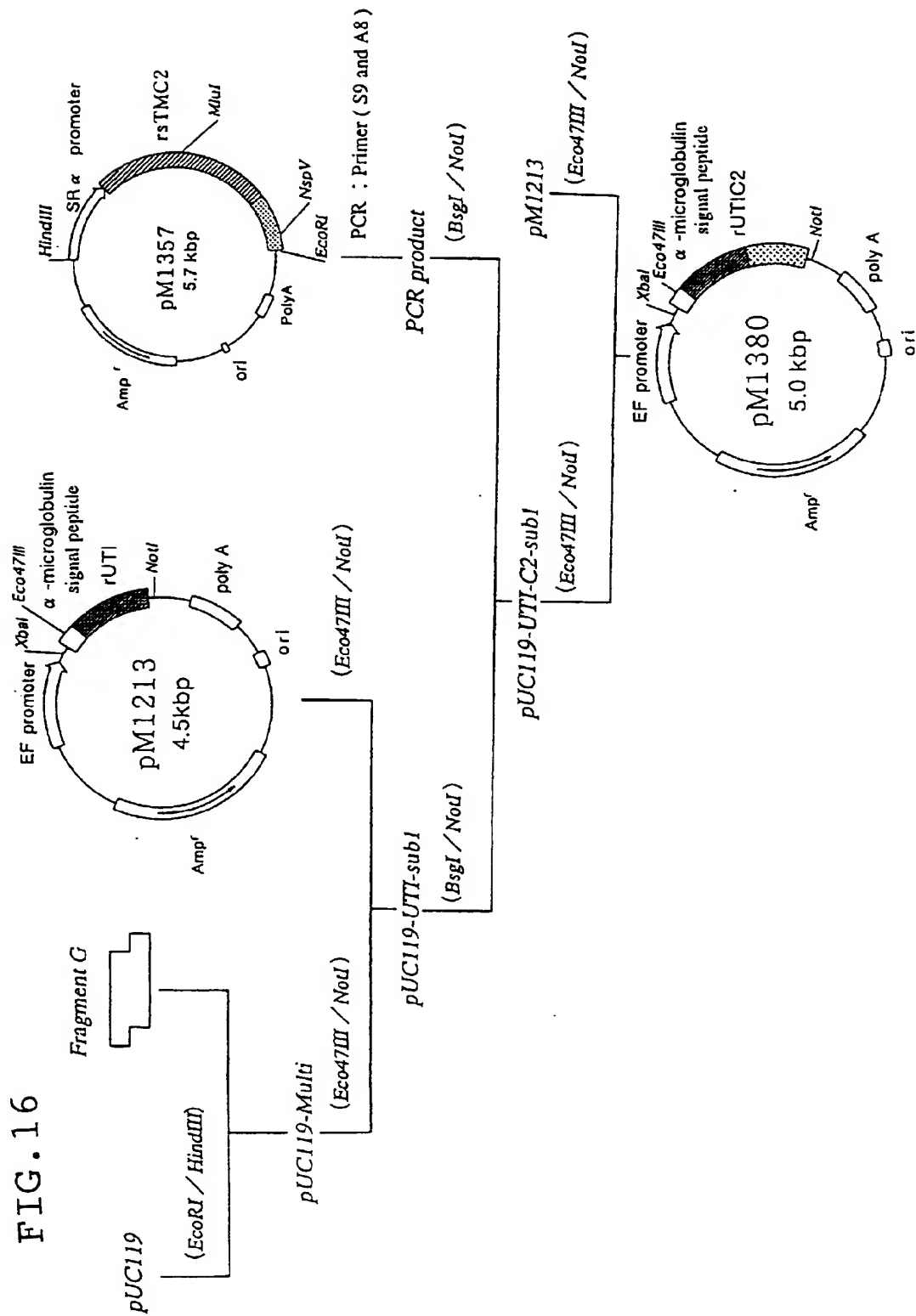


FIG. 17

Fragment H

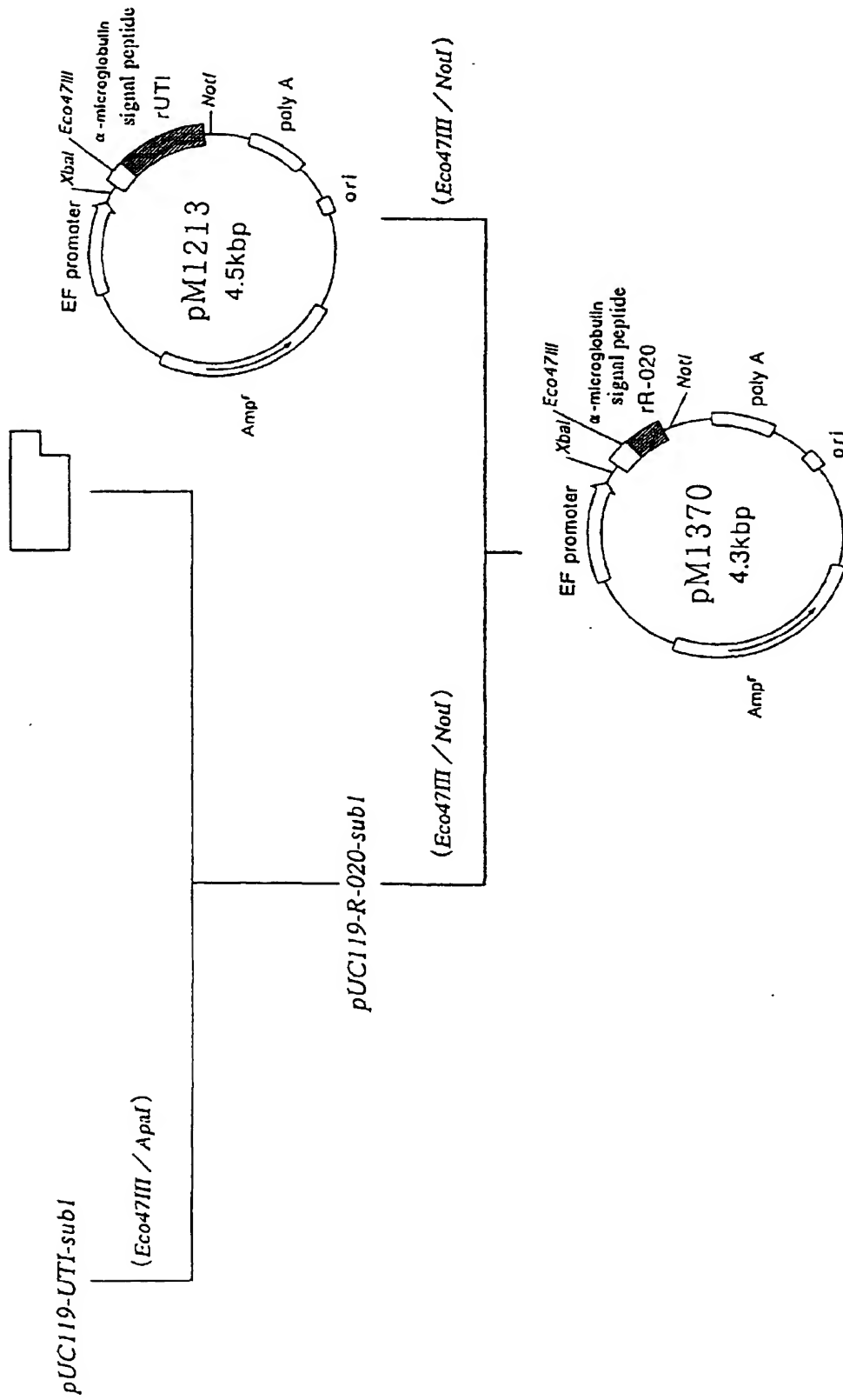


FIG. 18

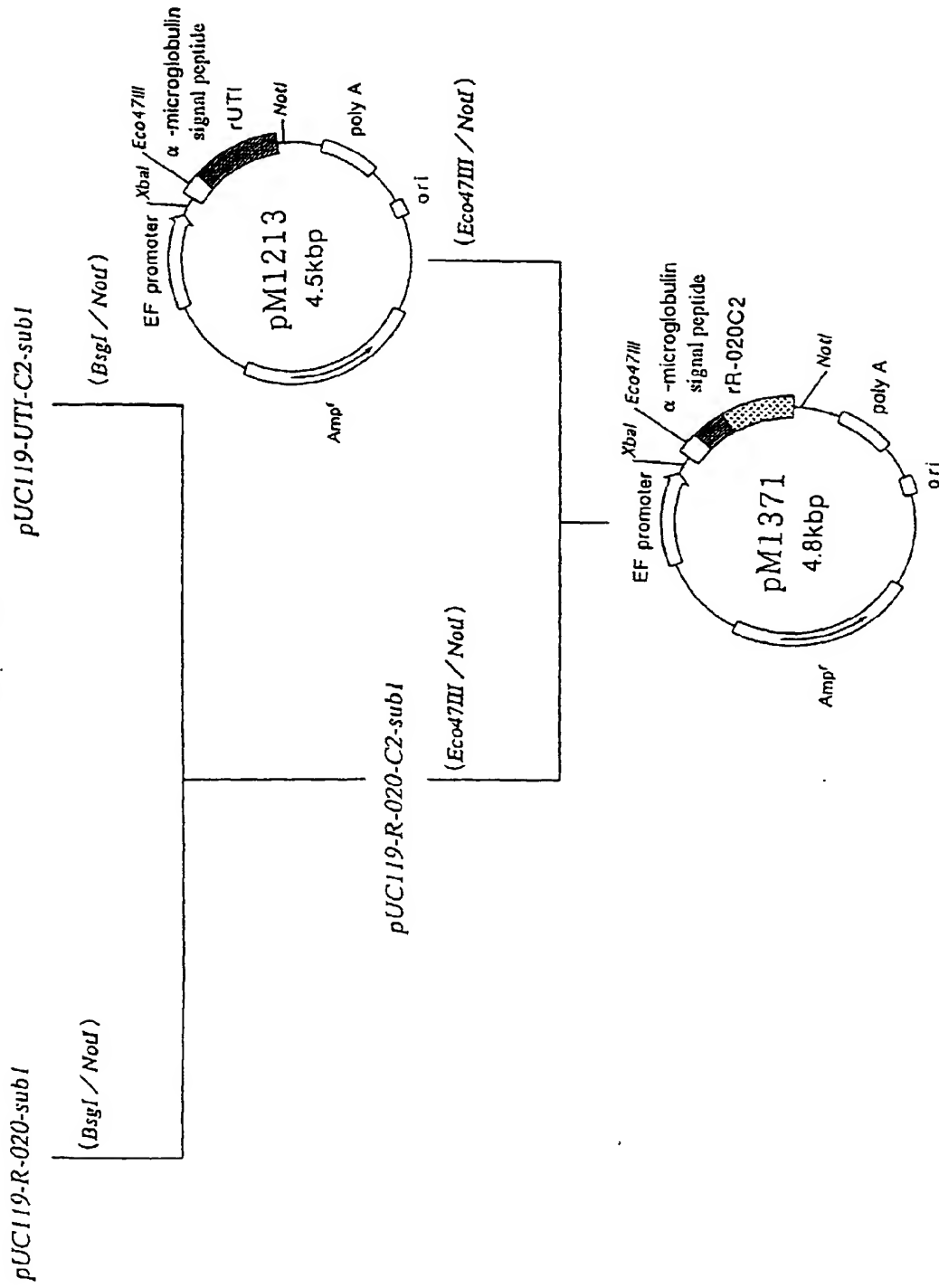


FIG. 19

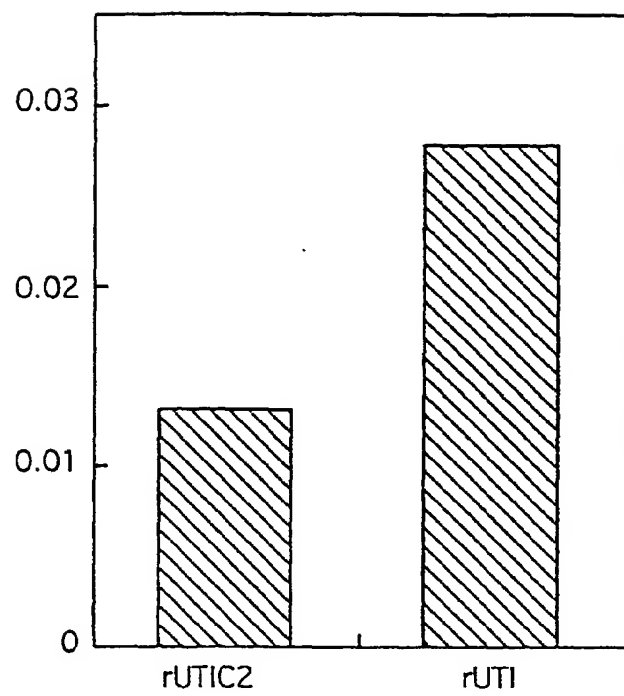


FIG. 20

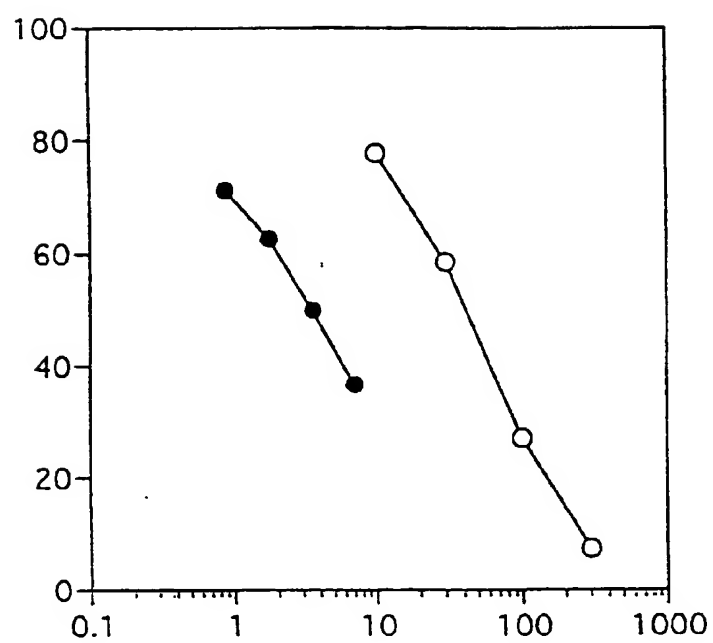


FIG. 21

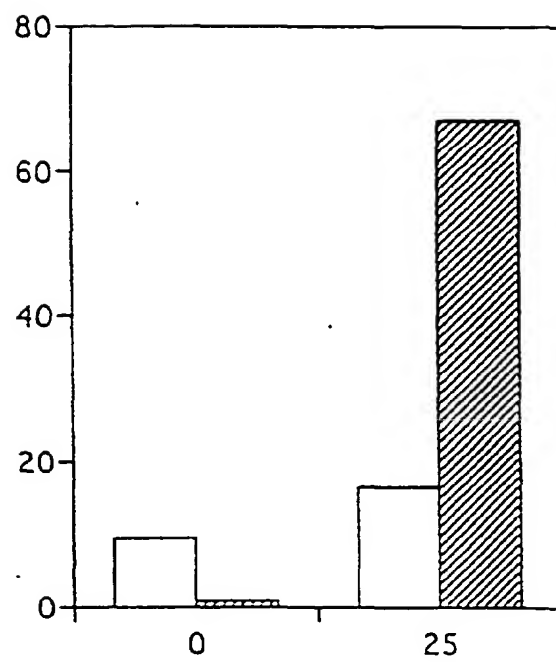
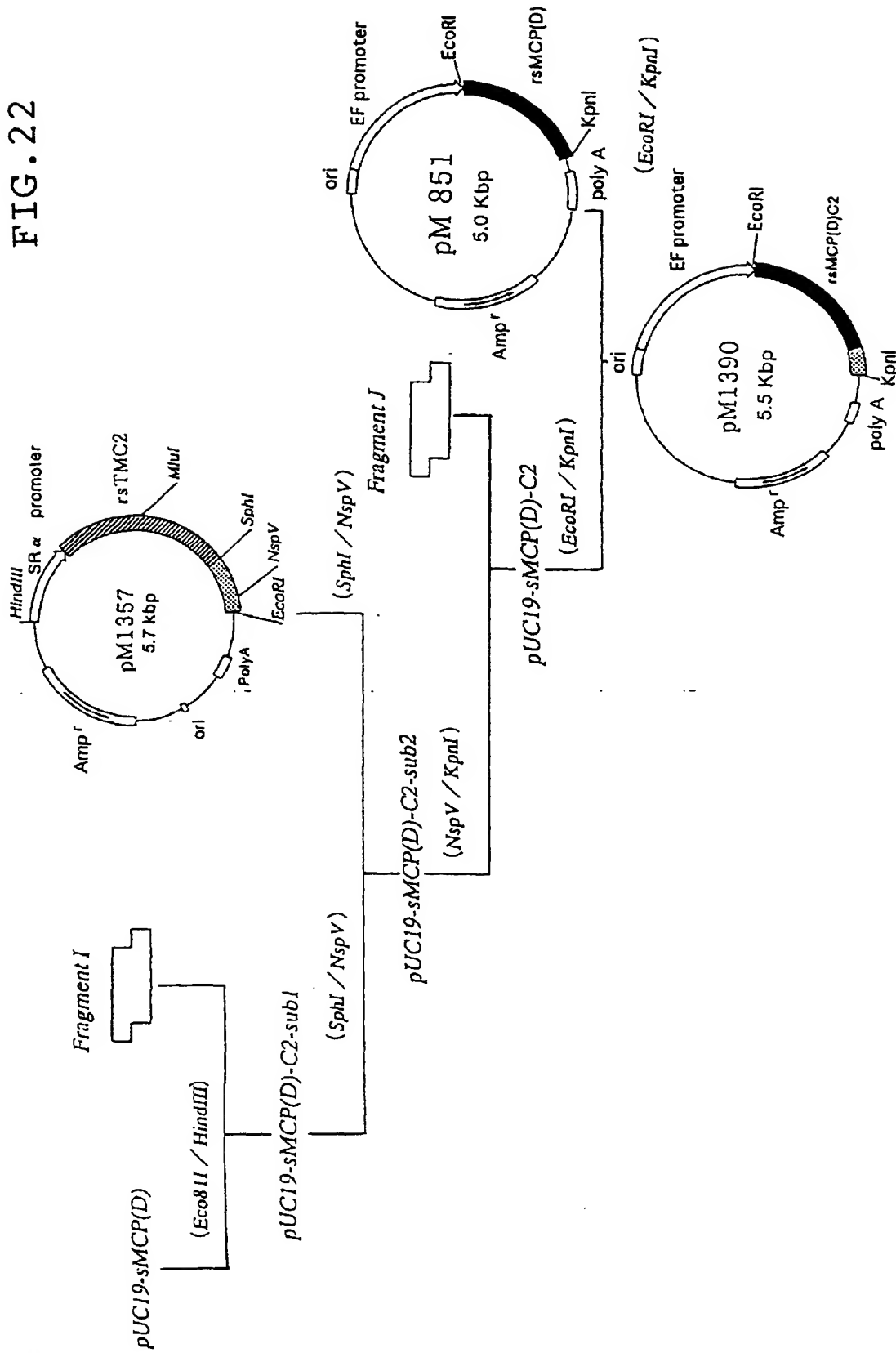


FIG. 22



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP98/00002

A. CLASSIFICATION OF SUBJECT MATTER Int.Cl ⁶ C07K19/00, C07K14/745, A61K47/48, C12P21/02, C12N15/12 // (C12P21/02, C12R1:91) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int.Cl ⁶ C07K19/00, C07K14/745, A61K47/48, C12P21/02, C12N15/12 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA (STN)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biochemistry, 34, 1995, Gary E. Gilbert et al., "Membrane-binding peptide from the C2 domain of factor VIII forms an amphipathic structure as determined by NMR spectroscopy" p.3022-3031	1-21
A	WO, 93/17122, A (MOCHIDA PHARM CO LTD), September 2, 1996 (02. 09. 96) & JP, 5-271288, A & EP, 581967, A	10-16, 19, 21
A	JP, 9-20677, A (MOCHIDA PHARM CO LTD), January 21, 1997 (21. 01. 97) (Family: none)	10-16, 19, 21
A	WO, 92/03149, A (SCHERING AG), March 5, 1992 (05. 03. 92) & EP, 544826, A & US, 5466668, A	10-16, 19, 21
A	WO, 88/05053, A (ASAHI KASEI KOGYO KK), July 14, 1988 (14. 07. 88) & JP, 1-6219, A & EP, 312598, A	10, 11, 15, 16, 19, 21
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search April 8, 1998 (08. 04. 98)		Date of mailing of the international search report April 21, 1998 (21. 04. 98)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)